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Report on current relevant biodegradation

and ecotoxicity standards

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Table of content

1	Publishable summary	8
2	Introduction	13
3	Freshwater aerobic aqueous environment.....	14
3.1	Biodegradation	14
3.1.1	OECD guidelines.....	17
3.1.2	European standards.....	27
3.1.3	International standards.....	28
3.1.4	American standards	37
3.2	Toxicity.....	46
3.2.1	Preparation of the test items.....	47
3.2.2	Test methods	50
3.3	Standard specifications	56
3.3.1	European specifications	56
3.3.2	International specifications	58
3.4	Labelling.....	59
3.4.1	Der Blaue Engel / Blue Angel.....	59
3.4.2	Swedish labelling scheme	60
3.4.3	Nordic Swan.....	61
3.4.4	EU Ecolabel	63
3.4.5	OK biodegradable WATER (Vinçotte)	71
3.5	Discussion and critical review.....	72
4	Marine aerobic aqueous environment.....	80
4.1	Biodegradation	80
4.1.1	OECD guidelines.....	80
4.1.2	International standards.....	82
4.1.3	American standards	83
4.2	Toxicity.....	86
4.2.1	Preparation of the test items.....	86
4.2.2	Test methods	86
4.3	Standard specifications	88
4.4	Labelling.....	90
4.5	Discussion and critical review.....	91
5	Anaerobic environment	93



5.1	Biodegradation	93
5.1.1	OECD guidelines.....	93
5.1.2	European standards.....	94
5.1.3	International standards.....	94
5.1.4	American standards	97
5.2	Toxicity.....	100
5.2.1	OECD guidelines.....	100
5.2.2	International standards.....	100
5.3	Standard specifications	101
5.4	Labelling.....	101
5.5	Discussion and critical review.....	102
6	Soil environment	104
6.1	Biodegradation	105
6.1.1	ASTM Standards for testing biodegradability of plastics in soil	106
6.1.2	ISO Standards for testing biodegradability of plastics and chemicals in soil ..	107
6.1.3	Testing method based on French norm for testing biodegradability of agricultural films in soil	110
6.1.4	OECD guidelines for testing biodegradability in soil	111
6.2	Toxicity.....	113
6.2.1	OECD guidelines.....	113
6.2.2	International standards.....	117
6.2.3	American standards	118
6.3	Standard specifications	119
6.3.1	ASTM Standards specifications for aerobically biodegradable plastics in soil	119
6.3.2	Specifications defined based on French norm for biodegradable agricultural films in soil.....	120
6.3.3	Belgian Royal Decree for Acceptance of Compostable and Biodegradable Plastic Materials	121
6.4	Labelling.....	122
6.4.1	VINÇOTTE Certest Products - OK biodegradable SOIL - Initial acceptance tests	122
6.5	Discussion and critical review.....	123
6.5.1	Biopolymers	133
6.5.2	Lubricants and solvents.....	134
6.5.3	Major discrepancies in the biodegradation test methods	134



7	Industrial composting environment	138
7.1	Biodegradation	139
7.1.1	ASTM Standards for testing biodegradability of biodegradable materials under municipal and industrial composting conditions	141
7.1.2	ISO Standards for testing biodegradability of plastics under municipal and industrial composting conditions	144
7.1.3	European Norms for testing biodegradability of plastics under municipal and industrial composting conditions	147
7.1.4	Other Norms for testing biodegradability of plastics under municipal and industrial composting conditions	147
7.2	Toxicity	148
7.3	Standard specifications	150
7.3.1	ASTM Standard specifications for characterising compostable plastic products and materials	151
7.3.2	ISO Standard specifications for characterising compostable materials	154
7.3.3	European Norms specifications for characterising compostable packaging, plastic products and materials	157
7.3.4	Belgian Royal Decree for Acceptance of Compostable and Biodegradable Plastic Materials	159
7.3.5	Australian Standard AS 4736:2006	160
7.3.6	Other national specifications for characterising compostable packaging, plastic products and materials	160
7.4	Labelling	162
7.4.1	VINÇOTTE Certest Products - OK compost - Initial acceptance tests	164
7.5	Discussion and critical review	165
7.5.1	Biodegradability	165
7.5.2	Disintegration	172
7.5.3	Industrial compostability	173
7.5.4	Home compostability	173
8	Relation towards REACH	174
9	Conclusion	176
10	References	180



List with abbreviations

ASTM:	American Society for Testing and Materials
BCF:	BioConcentration Factor
BOD:	Biochemical Oxygen Demand
CEN:	European committee for standardisation (Comité Européen de Normalisation)
CFU:	Colony-Forming Units
COD:	Chemical Oxygen Demand
DO:	Dissolved Oxygen
DOC:	Dissolved Organic Carbon
EC:	Effect Concentration
ECB:	Effective Composition to Biodegradation
ECHA:	European Chemicals Agency
EMPA:	Swiss Federal Laboratories for Materials Testing and Research
EU:	European Union
HRT:	Hydraulic Retention Time
IC:	Inorganic Carbon
ISO:	International Organization for Standardization
LC:	Lethal concentration
LOEC:	Lowest Observed Effect Concentration
MATC:	Maximal Acceptable Toxicant Concentration
MITI:	Ministry of International Trade and Industry (Japan)
MHC:	Moisture-Holding Capacity
MSW:	Municipal Solid Waste
NOEC:	No Observed Effect Concentration
OECD:	Organisation for Economic Co-operation and Development
PAH:	Polycyclic aromatic hydrocarbon
PCB:	Polychlorinated biphenyl



QSAR:	Quantitative Structure-Activity Relationship
REACH:	Registration, Evaluation and Authorisation of Chemicals
SCAS:	Semi-Continuous Activated Sludge
SOC:	Soluble Organic Carbon
SRT:	Sludge Retention Time (= sludge age)
ThIC:	Theoretical Inorganic Carbon
ThOD:	Theoretical Oxygen Demand
ThCO ₂ :	Theoretical carbon dioxide
TIC:	Total Inorganic Carbon
TS:	Total Solids
VFA:	Volatile Fatty Acids
WAF:	Water-Accommodated Fraction
WHC:	Water Holding Capacity
WSF:	Water-Soluble Fraction



1 Publishable summary

In this report the current biodegradation test methods in different environments (fresh water, marine environment, anaerobic environment, soil and compost) and the existing test procedures for evaluating environmental safety are reviewed. Existing difficulties and gaps in the current test methods are defined.

This review is focussed on the applicability of the test methods to bio-lubricants and bio-solvents. Consumers consider the “bio”-prefix often as a synonym of good for the environment. It should be avoided that the term “bio” refers to subjective non-measurable criteria (e.g. environmentally friendly, environmentally acceptable, etc.). Objective criteria should be selected with well-defined criteria.

In order to ensure transparent communication, specifications and labelling systems need to be developed. Standardised test methods form the cornerstones for specifications, which encompass well-defined pass criteria with regard to different characteristics (e.g. biodegradation, environmental safety, bio-based content, performance, etc.). These criteria are chosen in function of the objective of the specification. The specifications are designed to form the basic principle for labelling systems. Currently specifications, acceptance criteria and labelling systems are already clearly defined for compostable plastics and packaging, while this is only to a lesser degree the case for other environments (fresh water, marine environment, anaerobic environment and soil) and other products. Existing specifications and labelling systems for plastics, bio-lubricants and bio-solvents are reviewed per environment and difficulties and gaps in the existing specifications are defined.

Freshwater aerobic aqueous environment

Based on the literature review of the different biodegradation test methods in an aqueous aerobic freshwater environment it can be concluded that a sufficiently broad range of measurement techniques already exists. Not each test method is suitable to test bio-lubricants or bio-solvents due to the fact that bio-lubricants are often poorly water soluble and that bio-solvents are often volatile. Therefore specific biodegradation test methods need to be selected taking into account these characteristics. Special care should be given towards the addition of these substances (poorly water soluble or volatile) to the testing system and if required special addition techniques should be used. It also needs to be evaluated if the usual water-soluble reference materials (aniline, sodium benzoate, etc.) should be replaced by a poorly water soluble or a volatile alternative.

The review of the freshwater biodegradation test methods also revealed a few items, which should be further investigated in order to optimize the test methods. Among others influence of inoculum source (geographical variations, seasonal variations, etc.) on the biodegradability potential and variability of the results, interpretation of variability due to nitrification, necessity of the addition of a nitrification inhibitor, determination of the minimum amount of replicates, etc. should be further investigated.

With regard to environmental safety, it can also be concluded that a sufficiently broad range of testing methods towards freshwater organisms on different trophic levels (bacteria, algae, freshwater aquatic plants, crustacean and fish) already exists. For bio-lubricants and bio-



solvents, additional attention is especially needed towards the addition of poorly water soluble bio-lubricants and volatile bio-solvents to the testing systems as this can influence the test results.

The review revealed that specifications need to be developed towards bio-lubricants and bio-solvents. A technical report with useful recommendations for a specification towards bio-lubricants (CEN/TR 16227) and the specifications for the EU Ecolabel for lubricants can be taken as a guideline in order to develop a specification towards bio-lubricants. This should also be developed for bio-solvents.

A labelling system for bio-lubricants has been developed by different organisations, but no European or international labelling systems especially towards bio-solvents is developed yet. This should be developed taking into account parameters like biodegradability, environmental safety, minimum bio-based content, etc.

Work with regard to the above mentioned aspects will be performed in the framework of task 6.2 of KBBPPS.

Marine aerobic aqueous environment

From the literature review on the biodegradation test methods in a marine aerobic environment, it can be concluded that there exist considerably less biodegradation test methods when compared to a freshwater environment. However, a sufficiently broad range of methods exists in order to determine the biodegradation in a marine environment. Not all methods are suitable in order to evaluate the biodegradability of bio-lubricants and bio-solvents, but a suitable measurement technique can be selected taken into account the specific properties (volatility and/or solubility) of a bio-lubricant or a bio-solvent. Special care should be given towards the addition of these substances (poorly water soluble or volatile) to the testing system and if required special addition techniques should be used.

The review of the marine biodegradation test methods also revealed a few items, which should be further investigated in order to optimize the test methods. Among others the inoculum (natural seawater versus artificial seawater), the addition of nutrients, the difference between conditions in different parts of the sea (supralittoral, eulittoral, sublittoral benthic, deep sea benthic, pelagic & buried in the sediments), etc. should be further investigated.

With regard to marine environmental safety it can be concluded that less tests were developed when compared to the freshwater environment especially on OECD level. ISO and ASTM are already more progressive as more guidelines towards marine organisms were developed. The guidance documents towards the sample preparation and the interpretation of the results of toxicity tests for difficult substances, poorly water soluble substances and lubricants should be taken into account.

Currently no standard specifications towards more environmentally friendly alternatives for lubricants and solvents used in a marine environment are developed yet. These specifications need to be developed. This can be based on the EU Ecolabel for lubricants, which encompasses already marine applications.



Work with regard to the above mentioned aspects will be performed in the framework of task 6.2 of KBBPPS.

Anaerobic environment

Based on the review on the existing biodegradation standards in an anaerobic environment, it can be concluded that there exists a sufficiently broad range of standards in order to determine the degree of anaerobic biodegradation in aquatic environments, high-solids anaerobic-digestion environments and landfill environments. Suitable methods need to be selected for bio-lubricants and bio-solvents.

Lubricants and solvents can reach anaerobic aquatic environments in wastewater treatment plants, but high-solids anaerobic-digestion environments and anaerobic landfill environments are probably not considered as environments in which lubricants and/or solvents are often spilled or disposed. These standards are more suitable for biopolymers.

Toxicity towards anaerobic bacteria can be evaluated based on existing methods. These methods can be used for lubricants and solvents. The guidance documents towards the sample preparation and the interpretation of the results of toxicity tests for difficult substances, poorly water soluble substances and lubricants should be taken into account.

For biopolymers, which are degradable in anaerobic digestors, it might be necessary to evaluate if toxic residuals remain present in the produced digestate. Further research is needed in order to determine how this should be done.

Currently no standard specifications nor labelling systems are developed for products which are biodegradable in an anaerobic digester (e.g. biopolymers). This is mainly caused by the fact that there exists a wide variation in the construction and the operation of anaerobic digestion systems. The construction and operation systems can be divided into categories based on two parameters: (1) temperature (mesophilic and thermophilic) and (2) dry solids content (wet systems and dry systems). A standard specification should be developed, which includes criteria per operation system. This standard specification should form the basis for a new labelling system.

The labelling systems for lubricants do not refer to anaerobic environments in order to evaluate biodegradability and environmental safety. However, taken into account that a high percentage of ultimately aerobically biodegradable components needs to be present in the major part of the labelled products, it is expected that the labelled products will already be degraded before they come in contact with anaerobic environments.

Soil environment

A few international norms are available about testing biodegradability in soil. Their current weakness concerns their reliability in the cases of intentional incorporation of biodegradable materials in the soil under real conditions. Such a practice is widely used in agriculture and concerns the vast majority of applications where testing of biodegradation in soil is a key prerequisite with respect to both, environmental and food safety aspects. Considering the existing biodegradable plastics in agriculture and the effective life management of the



plastics in use at the agricultural field, only few norms have suitable tests that could be adapted for testing biodegradability in soil under real field conditions. The standardised criteria, parameters and testing methodologies for the characterization, labelling and validation of the agricultural plastic waste streams with respect to possible biodegradation in soil suggest that some major revisions are needed, before a new (i.e. revised) universal norm and improved standard testing methods become available for testing agricultural plastics for biodegradation under real, and highly variable, soil conditions. Based on the analysis of the different norms and their content it appears necessary to incorporate provisions for transferability of results to different soils, validation of test through a positive reference and set prerequisites for soil media. Furthermore, terminology and technical specifications vary and need to be harmonised. Long term biodegradation in soil prediction is another open issue (*Briassoulis and Dejean, 2010*). It is clarified though that there is no need for new testing method for biodegradation in soil. However, the existing standards for biodegradation in soil have to be improved and adapted in order to take into account the need for transferability of results to different soils under real field conditions. This goal should be achieved in a way that the standard testing method allows for repeatability of results by various laboratories. Another issue raised recently concerns the possibility to measure the possible production of new cell biomass or incorporating it into the humus that is not “measured” through the current testing methods.

An improved revised universal norm should be based on testing method(s) that include a well-documented range of several typical soil types and a well-defined range of conditions bracketing the majority of soil types and prevailing conditions, for a specific region. A basic requirement to characterise a product as “biodegradable product” is the necessary time for biodegradation. This is particularly important for agricultural applications where intentional incorporation in soil is the key motivation for the use of biodegradable products. Such a practice, if biodegradation rate is slow, may result into excessive accumulation of materials in the soil. The time use at the field will depend on the type of crop and on the farmer practices but it is common that the biodegradable plastics should have mineralised in the soil before the soil cultivation practices start for the next year crop. In addition, a range of grades for different biodegradation time under different latitudes or climates can be established, following the analogous procedure established by the standard for ageing of plastic films under different geographic areas (solar irradiance). For example in the French norm a set of grades is adopted to define the required biodegradation time of the different mulching films (*Briassoulis and Dejean, 2010*). In addition all relevant safety (e.g. heavy metals) and ecotoxicity requirements for soil biodegradable products should be met (as for example required by the French Norm, OK biodegradable SOIL).

Concerning bio-based lubricants, solvents etc, there is a need for an appropriate testing method that should be based on proper adaptation of testing methods for biodegradation of bio-based polymers in soil, combined with specifications and labeling analogous to those already available for biodegradable in soil plastics. Work in this direction will be performed in the framework of task 6.2 of KBBPPS and will be based on adaptation of existing standards for biodegradation in soil of biobased plastics.



Industrial composting environment

Many norms concerning testing of compostable plastics have been developed at national and international level. Some are about plastic materials others about products like packaging. The media and conditions of testing cover mainly the conditions designed for industrial composting facilities, and only a few concern home composting conditions (*Briassoulis et al. 2010*). Also, only a few of the existing norms will be suitable, after appropriate revisions, to be adapted to testing biodegradable/compostable agricultural plastic products under farm composting conditions. Farm composting involves techniques not foreseen by the industrial or home composting methods. Farm composting is particularly relevant to biodegradation of biobased materials used in agriculture and specifications differ depending on the cultivations (e.g. organic farming requirements are more strict according to the relevant legislation).

The terminology and the biodegradability validation criteria under composting conditions, such as the threshold percentages of biodegradation and disintegration, the time and temperature, and the ecotoxicity, differ to some degree for the various norms and standard testing methods. Criteria for the establishment of a new integrative norm for compostable plastics used in agricultural applications need to be defined (*Briassoulis et al. 2010*). Such a norm may include for example home composting and farm composting or only farm composting test methods and specifications where the last one may be based on the existing test methods adapted to practices and conditions of farm composting.

An industrial composting environment is probably not considered as an environment in which lubricants and/or solvents are often spilled or disposed. These standards are more suitable for biopolymers.



2 Introduction

In this report the current biodegradation test methods in different environments (fresh water, marine environment, anaerobic environment, soil and compost) and the existing test procedures for evaluating environmental safety are reviewed. Existing difficulties and gaps in the current test methods are defined.

This review is focussed on the applicability of the test methods to bio-lubricants and bio-solvents. Consumers consider the “bio”-prefix often as a synonym of good for the environment. It should be avoided that the term “bio” refers to subjective non-measurable criteria (e.g. environmentally friendly, environmentally acceptable, etc.). Objective criteria should be selected with well-defined criteria. Currently, the prefix “bio” for lubricants and solvents is often associated with biodegradability and/or with the natural origin of the resources, but as long as no clear specifications exist, the term “bio” can be the source of misleading information and confusion for the final consumer (CEN/TR 16227).

In order to ensure transparent communication, specifications and labelling systems need to be developed. Standardised test methods form the cornerstones for specifications, which encompass well-defined pass criteria with regard to different characteristics (e.g. biodegradation, environmental safety, bio-based content, performance, etc.). These criteria are chosen in function of the objective of the specification. The specifications are designed to form the basic principle for labelling systems (Figure 1). Currently specifications, acceptance criteria and labelling systems are already clearly defined for compostable plastics and packaging, while this is only to a lesser degree the case for other environments (fresh water, marine environment, anaerobic environment and soil) and other products. Existing specifications and labelling systems for plastics, bio-lubricants and bio-solvents are reviewed and difficulties and gaps in the existing specifications are defined.

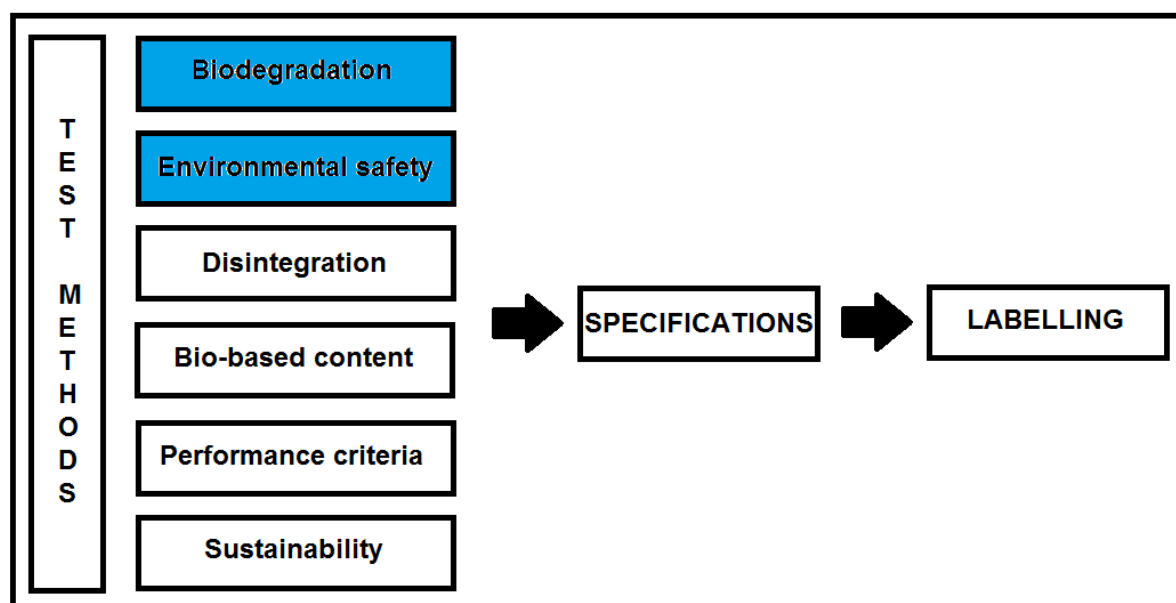


Figure 1. Relation between test methods, specifications and labelling systems.

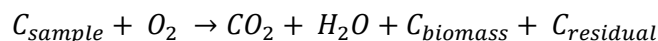


3 Freshwater aerobic aqueous environment

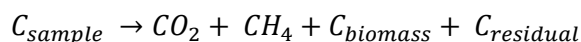
The review of the freshwater aerobic aqueous environment has been executed by OWS.

3.1 Biodegradation

Biodegradation can be defined as the breakdown or mineralisation of an organic material due to microbial and/or fungal activity. The availability of oxygen determines to which molecules the organic carbon is converted. If complete biodegradation takes place under aerobic conditions the organic carbon of the material will be converted to carbon dioxide and water (Equation 1), while under anaerobic conditions biogas, which is a mixture of carbon dioxide and methane, will be formed (Equation 2). The organic carbon of the material is in both cases also partly converted to new biomass ($C_{biomass}$) and it is possible that some carbon is not converted or remains present under the form of metabolites ($C_{residual}$) (De Wilde (2013)).



Equation 1. Biodegradation of carbon under aerobic conditions.



Equation 2. Biodegradation of carbon under anaerobic conditions.

The biodegradation rate is strongly affected by the environment in which the biodegradation takes place. An environment is determined by the moisture content, the temperature, the type of micro-organisms, the density of micro-organisms, etc. Figure 2 shows a classification with regard to aggressiveness of the aerobic environments.

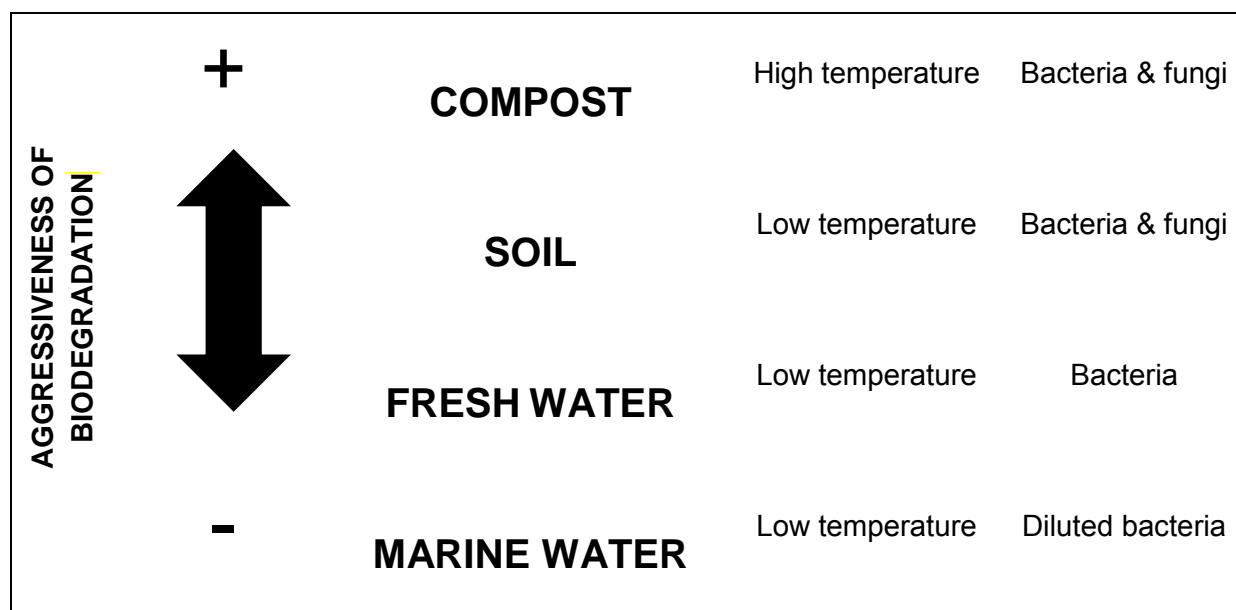


Figure 2. Relation between environment and aggressiveness of biodegradation.



Due to the differences with regard to aggressiveness between the environments and the typical nature of the milieu (solid ↔ aqueous), a biodegradability claim needs to be coupled to a specific well-defined environment. Therefore, test methods, which determine the biodegradability of a material, are always designed for a specific environment (fresh water, marine water, anaerobic conditions, soil or compost).

Biodegradation tests have their roots in the development of degradable synthetic surfactants in the mid-seventies. The first guidelines were developed by the Organization for Economic Cooperation and Development (OECD) in 1981. The OECD is an international organization in which representatives of 29 industrialised countries in North America, Europe and the Pacific, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. OECD has a leading role in the promotion of internationally acceptable methods for the testing of chemicals (industrial chemicals, pesticides, food additives, pharmaceuticals, etc.) for regulatory purposes. Afterwards also CEN (European committee for standardisation), ISO (International Organization for Standardization) and ASTM (American Society for Testing and Materials) developed biodegradability guidelines for organic chemicals and also for more complex materials (plastics, etc.). The major part of these standards is based on the principles of the OECD guidelines. Currently a trend towards internationalisation is observed: ISO standards are automatically transformed into national standards and the Vienna treaty guarantees that there exists an agreement between ISO and CEN standards (De Wilde (2005)).

Aerobic aquatic freshwater biodegradation tests form a prediction for the fate of chemicals in an aerobic aqueous environment, especially in the aerobic stages of wastewater treatment as the used inoculum is often activated sludge from a wastewater-treatment plant. Different parameters can be monitored during a test in order to determine the biodegradability of organic products:

- Dissolved Organic Carbon (DOC)
- Dissolved Oxygen (DO)
- Respirometry: carbon dioxide production
- Respirometry: oxygen consumption
- Inorganic Carbon (IC)

The choice of which method should be used for a given chemical depends largely on its physical properties (solubility, volatility and adsorptivity). DOC removal can be influenced by abiotic elimination, such as adsorption onto activated sludge or evaporation. Although the respirometric method based on CO₂ production gives the most direct evidence of oxidation of organic carbon during biodegradation, it can also underestimate the degree of biodegradation due to the fact that some carbon is converted to cell growth. Uptake of oxygen is an indirect measure for assessing biodegradability and such results can be misleading due to oxygen consumption caused by nitrification or caused by chemical reactions (e.g. oxidation of ethanol to acetic acid uses up oxygen but no carbon is removed, decarboxylation reactions cause the loss of carbon without the uptake of oxygen).



The biodegradation percentages based on CO₂ production and oxygen consumption are always lower when compared to biodegradation percentages based on DOC removal because the conversion of carbon to biomass. The percentage carbon used for biomass production varies between species of bacteria and between chemicals. Consequently biodegradation percentages based on CO₂ production and oxygen consumption will vary from test to test and from chemical to chemical (Painter, H.A. (1995)).

The major part of the standards are developed for “chemicals” or “organic compounds”, while a few standards are especially developed towards plastics and lubricants. No specific standards for solvents are currently available. The suitability of the measurement techniques towards the biodegradability determination of lubricants, which are often poorly soluble in water and consist of complex mixtures of chemicals, and solvents, which are often volatile, differs.

Especially methods based on DOC measurements are not suitable for non-soluble and volatile materials. Moreover tests which measure CO₂ production are done frequently in systems that are swept regularly with fresh, CO₂-free air to maintain the aerobic environment. Consequently, materials with a high vapour pressure, which are in the air space of the test medium will be removed (ASTM D 6006 (2011)). Therefore, volatile test compounds can only be tested in closed systems.



3.1.1 OECD guidelines

The OECD has developed several guidelines for testing of chemicals on biodegradation in an aerobic aqueous medium. As these guidelines are developed for chemicals (= a form of matter that has a constant chemical composition and which cannot be separated into components by physical separation methods (= without breaking chemical bonds)), these test methods are not always suitable in order to determine biodegradation of complex materials.

OECD does not provide explicit guidance with regard to biodegradability testing on poorly water-soluble substances (= substances with a water solubility < 100 mg/l). The only critical guidance provided is the applicability of a restricted range of analytical methods (OECD 301 B, C, D (±) and F and OECD 310) and the requirement of additional control vessels where emulsifiers, solvents and carriers are used. Whilst advocating the use of emulsifiers, solvents and carriers, none are specifically identified and no guidance is provided regarding the acceptable level. Consequently, numerous approaches of introducing the test substance can be applied, which make it difficult to identify a set of core acceptable or workable solutions (ECHA (2012)). For volatile substances analytical methods OECD 301 C (±), D and F (±) and OECD 310 are suitable (additional information: see chapter 3.1.1.1.).

OECD 301 and 310 are both designed in order to determine ready biodegradability, while OECD 302 determines inherent biodegradability. OECD 303 and 309 are both guidelines for simulation biodegradation tests in specific freshwater aquatic environments. An overview of the guidelines of OECD with regard to aerobic aqueous biodegradation is given in Table 1.

Table 1. Overview of the OECD guidelines with regard to biodegradation in aerobic aqueous environment.

Guideline	Adopted	Method	Description
OECD 301 Ready Biodegradability	Jul-17-1992	A	DOC Die-Away
		B	CO ₂ Evolution (Modified Sturm Test)
		C	Modified MITI ¹ Test (I)
		D	Closed Bottle
		E	Modified OECD Screening
		F	Manometric Respirometry
OECD 302 Inherent Biodegradability	May-12-1981	A	Modified SCAS Test
	Jul-17-1992	B	Zahn-Wellens / EMPA ² Test
	May-12-1981	C	Modified MITI Test (II)
OECD 303 Simulation Biodegradation Test	Jan-22-2001	A	Activated Sludge Units
		B	Biofilms
OECD 309 Simulation Biodegradation Test	Apr-13-2004		Aerobic Mineralisation in Surface Water
OECD 310 Ready Biodegradability	Mar-23-2006		CO ₂ in sealed vessels (Headspace Test)

¹ MITI = Ministry of International Trade and Industry, Japan.

² EMPA = Swiss Federal Laboratories for Materials Testing and Research.



The term **“ready biodegradability”** refers to an arbitrary classification of chemicals, which have passed certain specified screening tests for ultimate biodegradability. It is assumed that such substances will rapidly and completely biodegrade in aquatic environments under aerobic conditions. Ready biodegradability tests are no simulation tests, but tests for potential to biodegrade. Consequently using data from them to assess the performance of a substance within a particular route to the environment would be a misuse of the data and unwarranted extrapolation. The data of such tests state that chemicals passing the test do not offer a serious challenge to the metabolic capability of aerobic aquatic environments (given the presence of bacteria, nutrients, etc.) and that they would be readily degraded in the real environment (Painter, H.A. (1995)).

The duration of these tests is always 28 days, pre-exposure of the inoculum to the chemical is not allowed, the test substance is provided in a rather high concentration (2 to 100 mg/l) as the sole source of carbon for energy and growth and the amount of DOC in the test solution due to the inoculum should be kept as low as possible compared to the amount of DOC due to the test substance. The endogenous activity of the inoculum is corrected by running parallel blank tests with inoculum but without test substance.

The pass levels for ready biodegradability as prescribed by OECD 301 and OECD 310 are:

- 70 % removal of DOC
- Biodegradation > 60 % ThOD
- Biodegradation > 60 % ThCO₂
- Biodegradation > 60 % ThIC

The pass level is higher for methods based on the measurement of the residual sample (DOC), while the pass level is lower for methods, which are based on respirometric measurements. This is caused by the fact that the biodegradation percentage based on CO₂ production and oxygen consumption is always less than the percentage determined by DOC removal due to the bacterial metabolism. Some of the organic carbon of the test substance is biochemically oxidized and converted to CO₂, while other fractions are synthesized into new cellular material or into organic metabolic products. These fractions are not oxidised and do not contribute to the CO₂ production. The biomass formation is related to different factors (nature of test substance, bacterial species, etc.).

The pass levels need to be reached within a 10-d window, which starts when the degree of biodegradation has reached 10 % DOC, ThOD, ThCO₂ or ThIC and must end before day 28 of the test. If chemicals are classified as readily biodegradable, the need for other ecological parameters (e.g. bioaccumulation and ecotoxicity) may be reduced. As no complete biodegradation needs to be demonstrated, these pass levels (60 % or 70 %) for ready biodegradability are only reasonable for pure chemicals, but not necessarily for chemical mixtures or for chemicals containing significant proportions of impurities.

The term **“inherent biodegradability”** is less stringent when compared to ready biodegradability. The test procedures offer a higher chance of detecting biodegradation compared to tests for ready biodegradability. Inherent biodegradability refers to a classification of chemicals for which there is unequivocal evidence of biodegradation (primary



or ultimate) in any test of biodegradability. Such substances are considered not to be persistent and can be assumed to be degraded in the aquatic environment in the medium or long term (in wastewater treatment plants or in other environmental compartments). If an inherent test is negative, this could indicate the potential for persistence in the environment. In these tests the ratio biomass to food is shifted in favour of the biomass and the potential for adaptation is increased significantly.

OECD 302 A prescribes that test chemicals giving a result $> 20\%$ loss of DOC may be regarded as inherently biodegradable, whereas a result $> 70\%$ loss of DOC is evidence of ultimate biodegradability. No recommendations with regard to the interpretation of the results are given in guidelines OECD 302 B and OECD 302 C. The definitions mentioned in Annex B of ISO 7827 (2010) also mention that removal of 20% DOC, consumption of 20% ThOD or evolution of 20% ThCO₂ in any degradation test, with or without pre-exposure of the inoculum, indicates that the test substance in question is inherently biodegradable and non-persistent.

A **simulation test** reproduces, in the laboratory, defined environmental conditions as well as possible and gives more extensive and better information on the degradation behaviour of substances under simulated environmental conditions. A representative and low concentration of test substance is used in tests designed to determine the biodegradation rate constant whereas higher concentration are for analytical reasons normally used for identification and quantification of major transformation products.



3.1.1.1 Ready biodegradability

A brief overview of the measurement techniques for ready biodegradability is given in Table 2. OECD 301 recommends method D (closed bottle) for volatile compounds, but also methods C (MITI) and F (manometric respirometry) can be suitable. Methods B (CO₂ evolution), C (MITI) and F (manometric respirometry) are recommended for poorly soluble compounds, but also method D (closed bottle) can be suitable. OECD 310 can also be used for poorly soluble test substances on condition that good dispersion can be ensured and for volatile substances with a Henry's law constant up to 50 Pa.m³.mol⁻¹. Methods based on DOC measurements (A and E) can be affected by physico-chemical processes such as adsorption, volatilization, precipitation and hydrolysis. The method based on the determination of evolved CO₂ (B), can be affected by the formation of inorganic carbon.

Table 2. Overview of measurement techniques of ready biodegradability test methods (OECD).

Method	Measurement technique
301 A	Test item (10-40 mg DOC/l) in an inoculated mineral medium is aerated at 22°C ± 2°C. Degradation is followed by DOC analysis. Degree of biodegradation is calculated by expressing the concentration of DOC removed (after correction for blank).
301 B	Test item (10-20 mg DOC/l) in an inoculated mineral medium is aerated with CO ₂ free air at 22°C ± 2°C. Degradation is followed by determining the CO ₂ produced. The produced CO ₂ is trapped in Ba(OH) ₂ or NaOH and is measured by titration of the residual hydroxide or as inorganic C. Degree of biodegradation is calculated by expressing produced CO ₂ (after correction for blank) as a percentage of ThCO ₂ .
301 C	Test item (100 mg/l) in a mineral medium inoculated with specially grown micro-organisms is stirred at 25°C ± 1°C. Evolved CO ₂ is absorbed by soda lime and oxygen uptake is measured by an electrolytic BOD meter or respirometer. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD.
301 D	Test item (2-5 mg/l) in a mineral medium inoculated with relatively small number of micro-organisms is kept in full, closed bottles at 22°C ± 2°C. Degradation is followed by analysis of dissolved oxygen. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD.
301 E	Test item (10-40 mg DOC/l) in an inoculated (relatively low concentration of micro-organisms) mineral medium is aerated at 22°C ± 2°C. Degradation is followed by DOC analysis. Degree of biodegradation is calculated by expressing the concentration of DOC removed (after correction for blank).
301 F	Test item (100 mg/l) in an inoculated mineral medium is stirred at 22°C ± 2°C. Evolved CO ₂ is absorbed by solution of KOH or other suitable absorbent and oxygen uptake is measured by a suitable respirometer. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD.
310	Test item (20 mg C/l) in an inoculated mineral medium is shaken in a sealed bottle with a headspace of air at 20°C ± 1°C. The CO ₂ evolution is determined by measuring the IC produced in the test bottles in excess of that produced in the blank vessels. The IC is measured by (A) acidification to pH < 3 or (B) conversion of CO ₂ from the headspace to carbonate. The extend of biodegradation is expressed as a percentage of ThIC (after correction for the blank).



An overview of the different inoculum sources as prescribed by OECD 301 and OECD 310 is given in Table 3. Pre-exposure (= pre-incubation of an inoculum in the presence of the test substance with the aim of enhancing the ability of the inoculum to degrade the test substance) is not allowed in order to determine ready biodegradability. OECD 310 gives more specifications with regard to the amount of colony-forming units (10^2 to 10^5 CFU per ml in the final mixture).

Table 3. Overview of inoculum sources of ready biodegradability test methods (OECD).

Source of inoculum	301 A	301 B	301 C	301 D	301 E	301 F	310
Activated sludge from aeration tank	X	X				X	X
Sewage effluent	X	X		X	X	X	X
Surface water	X	X		X		X	X
Soil extract	X	X				X	X
Mixture of inoculum from at least 10 sites, after at least 1 month in sludge unit			X				
Mixture of sources	X	X				X	X

The prescribed amount of the different series is given in Table 4. No exact amount of replicates is given for OECD 310 as the number of bottles depends on the frequency of analysis and the test duration. It is recommended that triplicate bottles need to be analysed at least weekly and at the end of the test also at least five bottles from the blank series, the reference series and the test series need to be analysed.

Table 4. Amount of replicates as prescribed by ready biodegradability test methods (OECD).

Method	Blank series	Reference series	Test series	Abiotic sterile control ³	Adsorption control ⁴	Toxicity control ⁵
301 A	2	1	2	1 (Preferably)	1 (Preferably)	1 (Preferably)
301 B	2	1	2	1 (Preferably)	-	1 (Preferably)
301 C	1	1	3	1	-	-
301 D	10	10	10	-	-	6 (If needed)
301 E	2	1	2	1 (Preferably)	1 (Preferably)	1 (Preferably)
301 F	2	1	2	1 (Preferably)	-	1 (Preferably)
310	Min. 17	Min. 17	Min. 17	Min. 8 (If needed)	-	Min. 8 (If needed)

³ Series containing test substance and sterilizing agent.

⁴ Series containing test substance, inoculum and sterilizing agent.

⁵ Series containing test substance, reference compound and inoculum.



In order to check *the validity of the test procedure*, OECD 301 prescribes that a reference compound, which meets the criteria for ready biodegradability needs to be tested in parallel. OECD 301 proposes aniline, sodium acetate or sodium benzoate as reference materials (Figure 3). OECD 310 refers to aniline, sodium benzoate or ethylene glycol for water-soluble test substances and to 1-octanol for poorly soluble test substances (Figure 4). The validity criteria depend on the used method (Table 5), but OECD 301 prescribes that the test is only considered valid if the difference of extremes of replicate values of removal of test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is < 20 %.

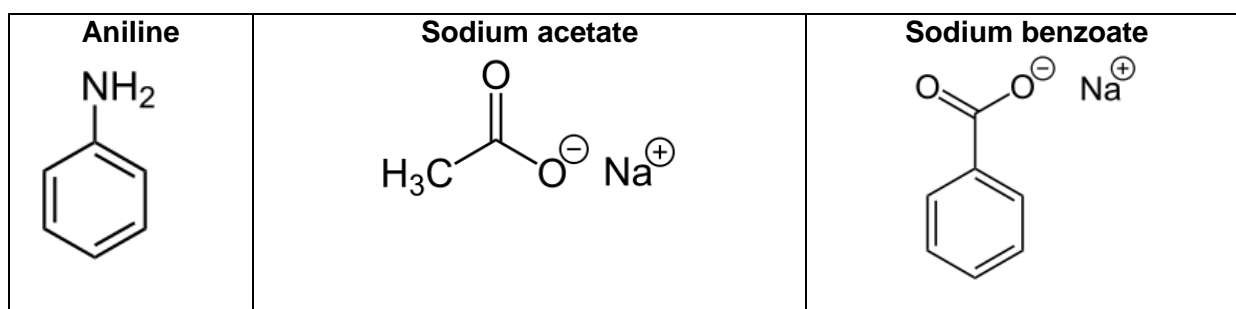


Figure 3. Structural formula of the reference compounds as described in OECD 301.

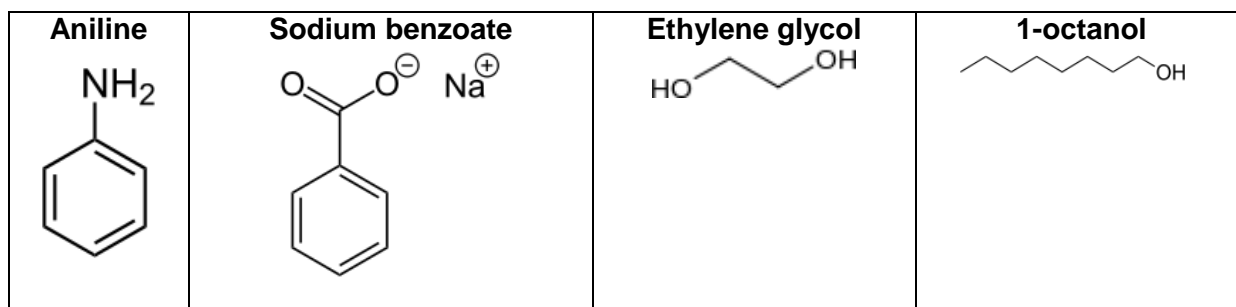


Figure 4. Structural formula of the reference compounds as described in OECD 310.

Table 5. Validity criteria as prescribed by ready biodegradability test methods (OECD).

Method	Validity criteria
301 A	Reference > 70 % DOC removal (after 14 days)
301 B	Reference > 60 % ThCO ₂ (after 14 days) IC content of test substance suspension < 5 % TC (at start) CO ₂ blank inoculum < 40 mg CO ₂ /l (certainly < 70 mg CO ₂ /l) (after 28 days)
301 C	Aniline > 40 % ThOD (after 7 days) & > 65 % ThOD (after 14 days) O ₂ blank inoculum < 60 mg O ₂ /l (after 28 days)
301 D	Reference > 60 % ThOD (after 14 days) O ₂ depletion in blank < 1.5 mg dissolved O ₂ /l (after 28 days) Residual O ₂ in test bottles > 0.5 mg/l (during entire test)
301 E	Reference > 70 % DOC removal (after 14 days)
301 F	Reference > 60 % ThOD (after 14 days) O ₂ blank inoculum < 60 mg O ₂ /l (after 28 days)
310	Reference > 60 % ThIC (after 14 days) TIC in the blank controls < 3 mg C/l (at end)



3.1.1.2 Inherent biodegradability

A brief overview of the measurements techniques for inherent biodegradability is given in Table 6, while Table 7 shows an overview of the inoculum sources. The minimum amount of replicates is given in Table 8. OECD 302 A “Inherent Biodegradability: Modified SCAS Test” is only applicable to test items, which are soluble in water (≥ 20 mg DOC/l) and have a negligible vapour pressure. Also OECD 302 B “Zahn-Wellens/EMPA Test” is also only applicable to non-volatile soluble organic test items (≥ 50 mg DOC/l). Consequently, these guidelines cannot be used in order to determine the inherent biodegradability of volatile substances or poorly water soluble substances. Test method OECD 302 C “Modified MITI Test (II)” is only applicable for chemicals with a negligible vapour pressure, but this method can be adapted for volatile chemicals using a modified BOD-meter. Moreover, this method can be applied for non-soluble test items.

Table 6. Overview of measurement techniques of inherent biodegradability test methods (OECD 302).

Method	Measurement technique
302 A	Test item (20 mg C/l) is exposed to high concentrations of micro-organisms over a long period (several months) in an aeration unit. Each day aeration is stopped, sludge is allowed to settle and supernatant liquor is removed. Remaining sludge is then again mixed with test item and sewage and the cycle is repeated. Biodegradation is followed by DOC measurements of the supernatant liquor.
302 B	Test item (50-400 mg DOC/l) is agitated and aerated in an inoculated (large amount of activated sludge) mineral medium at 20°C – 25°C. Degradation is followed by DOC analysis. Degree of biodegradation is calculated by expressing the concentration of DOC removed (after correction for blank).
302 C	Test item (30 mg/l) in a mineral medium inoculated with specially grown micro-organisms is stirred at 25°C \pm 2°C. Evolved CO ₂ is absorbed by soda lime and oxygen uptake is measured (BOD meter). Biodegradability is calculated on the basis of BOD and supplemental chemical analysis (DOC, concentration of residual chemicals, etc.).

Table 7. Overview of inoculum sources of inherent biodegradability test methods (OECD 302).

Source of inoculum	A	B	C
Activated sludge	X	X	
Mix of different sources (waste water treatment plants, soil extracts, river water, etc.)		X	
Mixture of inoculum from at least 10 sites (city sewage plant, industry sewage plant, rivers, lakes, marshes, sea,...), after at least 1 month in sludge unit⁶			X

Table 8. Amount of replicates as prescribed by inherent biodegradability test methods (OECD 302).

Method	Blank series	Reference series	Test series	Additional series
302 A	1	-	1	-
302 B	1 or 2	1	1 or 2	-
302 C	1	1	3	1 (water + chemical)

⁶ In the sludge unit the inoculum is regularly fed with synthetic sewage containing glucose, peptones and monopotassium phosphate.



No specific reference substrates are recommended in OECD 302 A, but data on several compounds (4-acetyl amino-benzene sulphonate, tetra propylene benzene sulphonate, 4-nitrophenol, diethylene glycol, aniline and cyclopentane tetra carboxylate) used in ring tests are provided in order to calibrate the method from time to time and in order to permit comparison of results. In order to check the functional capability of the activated sludge, OECD 302 B prescribes that a reference compound of known biodegradability, should be run in parallel with each test series. Following reference materials are recommended: ethylene glycol, diethylene glycol, lauryl sulfonate and aniline (Figure 5). OECD 302 C refers to the same reference materials as prescribed by OECD 301 (aniline, sodium acetate or sodium benzoate) (Figure 3).

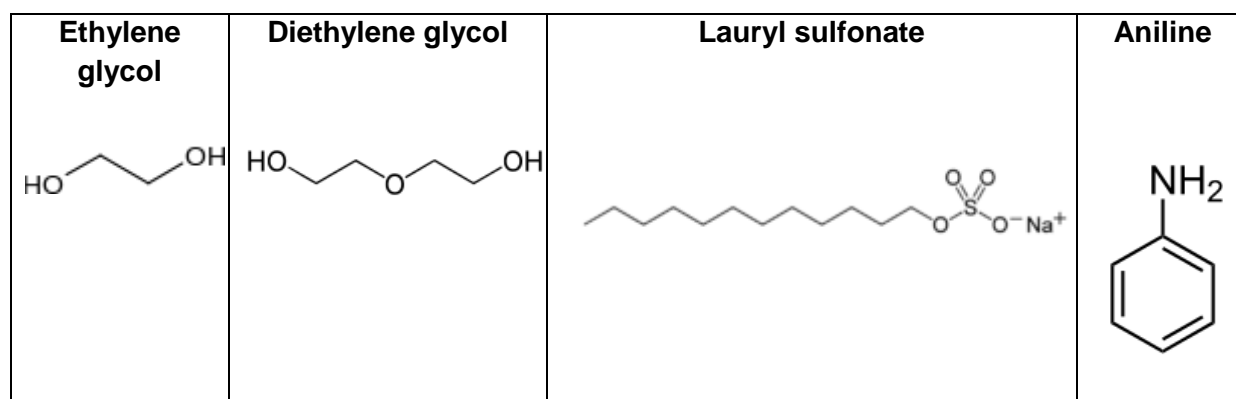


Figure 5. Structural formula of the reference compounds as described in OECD 302 B.

An overview of the validity criteria as prescribed by the OECD 302 guidelines is given in Table 9.

Table 9. Validity criteria as prescribed by inherent biodegradability test methods (OECD 302).

Method	Validity criteria
302 A	-
302 B	Reference > 70 % DOC removal (after 14 days)
302 C	Aniline > 40 % (after 7 days) & > 65 % (after 14 days) Recovery rate of test compounds in the blank tests with water only > 10 %



3.1.1.3 Simulation biodegradation tests

Guideline OECD 303 A simulates an activated sludge unit, while OECD 303 B simulates waste water treatment involving biofilms (percolating or trickling filters, rotation biological contactors, fluidised beds). Guideline OECD 309 simulates the biodegradation in surface water ("pelagic test") or in turbid surface water which, for example, might exist near a water/sediment interface ("suspended sediment test"). A brief overview of the measurements techniques is given in Table 10, while Table 11 shows an overview of the inoculum sources.

Table 10. Overview of measurement techniques of simulation biodegradability test methods (OECD).

Method	Measurement technique
303 A	Test item (10-20 mg DOC/l) is added together with an easily biodegradable organic medium (domestic sewage is recommended) to the influent of a continuously operating test system simulating an activated-sludge process (activated sludge plant model = Husmann apparatus or porous pot) with a mean hydraulic retention time (HRT) of 6 h and a mean sludge retention time (SRT) of 6 d to 10 d at 20°C – 25°C. Effluent samples are analysed for DOC. The difference between DOC values in effluent of test and control unit compared to the influent concentration is used to determine the degree of elimination of the test compound. Duration is 12 weeks.
303 B	Test item (10-20 mg C/l) is added together with an easily biodegradable organic medium (domestic sewage is recommended) to the internal surface of a slowly rotating tubular reactor with a residence time of 125 ± 12.5 sec. for the feed in a clean tube (maximum film: retention time ± 30 min) at 22°C ± 2°C. A layer of micro-organisms is built up on the internal surface. Effluent samples are analysed for DOC. The difference between DOC values in effluent of test and control unit compared to the influent concentration is used to determine the degree of elimination of the test compound. Duration is 12 weeks.
309	Test item (2 different concentration levels, which differ from each other by a factor of 5 to 10 & both < 100 µg/l & lowest concentration < 10 µg/l) is added to stirred natural surface water (= pelagic test) or to surface water with suspended solids/sediment of 0.01 to 1 g/l (= suspended sediment test) at field temperature or at a standard temperature of 20°C - 25°C and serves as secondary substrate (= first order biodegradation kinetics). The degradation is followed by the determination of the residual concentration of the test compound by radiotracer technique (= ¹⁴ C-labelling of most stable part of the molecule and liquid scintillation counting) or by chemical analysis. A secondary objective of the test is to obtain information on the primary degradation and the formation of major transformation products. Higher test concentrations may be used in this case and less stable parts of the molecule may be labelled. The duration of the test is normally 60 days, but the test may be extended up to 90 days.

Table 11. Overview of inoculum sources of simulation biodegradability test methods (OECD).

Source of inoculum	303 A	303 B	309
Activated sludge	X		
Effluent	X		
Surface water	X		X
Airborne inoculation		X	
Settled sewage		X	
Surface water & sediment			X



The prescribed amount of replicates is given in Table 12.

Table 12. Amount of replicates as prescribed by simulation biodegradability test methods (OECD).

Method	Blank series	Reference series	Test series	Sterile control ⁷	Solvent control
303 A	1	1 (occasionally)	1	-	-
303 B	1	1 (occasionally)	1	-	-
309	1	2	(2 ×) (2 ×) 2 ⁸	1 or 2	2

OECD 303 A and OECD 303 B recommend both that it is useful to test occasionally substances (adipic acid, 2-phenyl phenol, 1-naphthol, diphenic acid, 1-naphthoic acid) (Figure 6) whose behaviour is known simultaneously when test substances are investigated. In order to ensure that the microbial activity of the test water is within certain limits (= water contains an active microbial population), OECD 309 prescribes that a substance, which is easily degraded under aerobic conditions, should be used as reference substrate (aniline or sodium benzoate) (Figure 3).

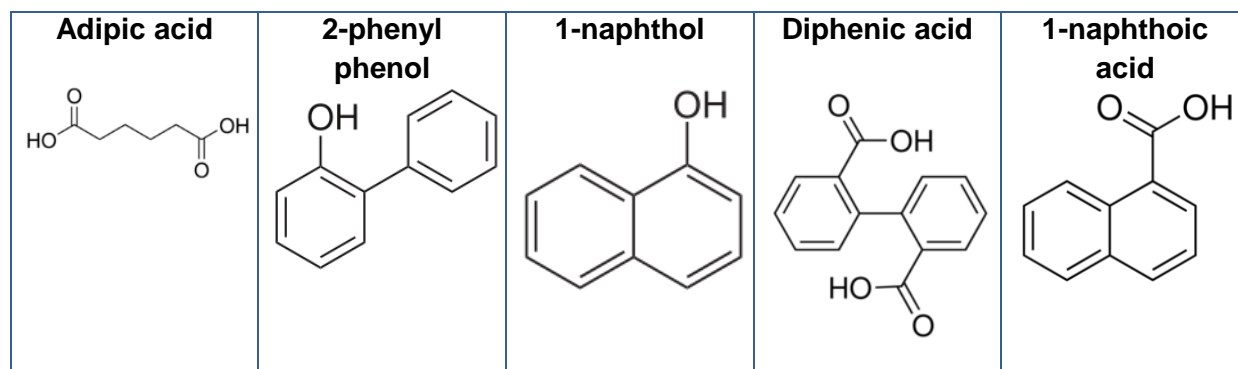


Figure 6. Structural formula of the reference compounds as described in OECD 303.

An overview of the validity criteria is given in Table 13.

Table 13. Validity criteria as prescribed by simulation biodegradability test methods (OECD).

Method	Validity criteria
303 A	DOC (or COD) degradation in control units > 80 % (after 2 weeks) No unusual observations Biodegradation of readily biodegradable reference substances > 90 % Ammonia-N < 1 mg/l & nitrite-N < 2 mg/l (test under nitrifying conditions)
303 B	DOC (or COD) degradation in control units > 80 % (after 2 weeks) No unusual observations Biodegradation of readily biodegradable reference substances > 90 % Difference between duplicate values < 5 %
309	Reference substrate needs to degrade sufficiently Total recovery at end: 90 % - 110 % (radiolabelled test items) Initial recovery at start: 70 % - 110 % (non-labelled test items)

⁷ Series containing sterilized test water for examining abiotic degradation.

⁸ Two replicates for each test concentration and also two replicates for each concentration for mass balance calculation.



3.1.2 European standards

For organic compounds and plastic materials CEN refers to existing ISO standards. CEN has developed 2 standards for packaging materials. EN 14047 & EN 14048 mention that biodegradability of packaging materials and its constituents must be determined in line with ISO 14852 and ISO 14851, respectively. An overview of the standards is given in Table 14.

Table 14. Overview of the EN standards with regard to biodegradation in aerobic aqueous environment.

Standard	Description
EN ISO 7827 (1995)	Water quality – Evaluation in an aqueous medium of the “ultimate” aerobic biodegradability of organic compounds – Method by analysis of dissolved organic carbon (DOC) (ISO 7827:1994)
EN ISO 9408 (1999)	Water quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer (ISO 9408:1999)
EN ISO 9439 (2000)	Water quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – Carbon dioxide evolution test (ISO 9439:1999)
EN ISO 9887 (1994)	Water quality – Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium – Semi-continuous activated sludge method (SCAS) (ISO 9887:1992)
EN ISO 9888 (1999)	Water quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – Static test (Zahn-Wellens method) (ISO 9888:1999)
EN ISO 10634 (1995)	Water quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium (ISO 10634:1995)
EN ISO 10707 (1997)	Water quality – Evaluation in an aqueous medium of the “ultimate” aerobic biodegradability of organic compounds – Method by analysis of biochemical oxygen demand (closed bottle test) (ISO 10707:1994)
EN ISO 11733 (2004)	Water quality – Determination of the elimination and biodegradability of organic compounds in an aqueous medium – Activated sludge simulation test (ISO 11733:2004)
EN ISO 14593 (2005)	Water quality – Evaluation of the ultimate aerobic biodegradability of organic compounds in aqueous medium – Method by analysis of inorganic carbon in sealed vessels (CO ₂ headspace test) (ISO 14593:1999)
EN ISO 14851 (2004)	Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium – Method by measuring the oxygen demand in a closed respirometer (ISO 14851:1999)
EN ISO 14852 (2004)	Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium – Method by analysis of evolved carbon dioxide (ISO 14852:1999)
CEN ISO/TR 15462 (2009)	Water quality – Selection of tests for biodegradability (ISO/TR 15462:2006)
EN 14047 (2002)	Packaging – Determination of the ultimate aerobic biodegradability of packaging materials in an aqueous medium – Method by analysis of evolved carbon dioxide
EN 14048 (2002)	Packaging – Determination of the ultimate aerobic biodegradability of packaging materials in an aqueous medium – Method by measuring oxygen demand in a closed respirometer



3.1.3 International standards

So far ISO has not developed specific standards with regard to the aerobic biodegradation in an aqueous medium for solvents or lubricants. In 1995 ISO concluded that the development of a single method for evaluating the biodegradability of poorly water-soluble organic substances might not be realized in the near future. Therefore, ISO proposed a series of methods where the final selection was based on a judgement of the physic-chemical properties of the test substance (ECHA (2012)). These preparation methods are described in ISO 10634 (1995) "Water quality – Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium". Four preparation techniques are described in this standard:

- Direct addition
 - a. Test component is weighted and direct addition
 - b. Using an inert support (for example: microscope slides)
 - c. Using a volatile solvent, which is removed prior to testing
- Ultrasonic dispersion
- Adsorption on an inert support (for example: silica gel or glass fibre filters)
- Dispersions or emulsions with an emulsifying agent

After the pre-treatment, the biodegradation in an aerobic aqueous medium can be tested using the standard methods, but methods based on DOC measurements are normally not suitable.

ISO has developed different test methods in order to determine the biodegradability of organic compounds and plastics in an aerobic aqueous environment (Table 15). These standards are mainly based on the principles of the OECD guidelines, but the international standards are often more precise and clearer when compared to the OECD guidelines. The major part of the international standards are related to the determination of the biodegradability of organic compounds in an aqueous medium. Only 2 standards are related to the biodegradability of plastic materials (ISO 14851 and ISO 14852). These standards (ISO 14851 and ISO 14852) are based on the same principles as ISO 9439 and ISO 9408, respectively.

The international biodegradability standards are based on the same principles, but due to differences in microbial density, test item concentration and test duration, these standards have not all an equal biodegradation potential.

Test method ISO 14592 is characterised by low microbial densities and very low test item concentrations ($< 200 \mu\text{g/l}$) as this method is developed in order to evaluate the biodegradation of substances at low environmentally realistic concentrations in the aquatic environment. ISO 14592-1 is a batch test simulating standing water bodies (lakes or ponds), while ISO 14592-2 is a dynamic test simulating flowing waters (rivers).

ISO 10707 (closed bottle test) is also characterised by a rather low microbial density and a low test item concentration (2 mg/l). Moreover these vessels are not stirred nor aerated. Therefore, the biodegradation potential of this method is also rather low. This method is especially developed for volatile and inhibitory test compounds.



Test methods ISO 7827 (DOC), ISO 9408 (oxygen consumption), ISO 9439 (CO₂ production), ISO 10708 (two-phase closed bottle) and ISO 14593 (CO₂ headspace test) are all characterised by a higher biodegradation potential when compared to ISO 14592 and ISO 10707 as the microbial density and the test item concentration are considerably higher. These guidelines are comparable to the OECD guidelines for ready biodegradability (OECD 301 & OECD 310). In spite of the fact that the biodegradation potential of these methods should be rather comparable, it is still possible that different results are obtained. This can be caused by differences in measurement techniques.

Test methods ISO 9887 (SCAS test) and ISO 9888 (Zahn-Wellens test) have a high inoculum concentration and may be extended further than the usual 28 days. The conditions as prescribed in these methods are optimal in order to allow the maximum biodegradation value of a test item. These methods can be used to determine the intrinsic biodegradation of chemicals, which corresponds the OECD guidelines for inherent biodegradability.

ISO 11733 (activated sludge simulation test) is characterised by the highest inoculum concentration, as this continuous test simulates a wastewater treatment plant including nitrification and denitrification techniques.



Table 15. Overview of the ISO standards with regard to biodegradation in aerobic aqueous environment.

Standard	Description
ISO 7827 (2010)	Water quality – Evaluation of the “ready”, “ultimate” aerobic biodegradability of organic compounds in an aqueous medium – Method by analysis of dissolved organic carbon (DOC)
ISO 9408 (1999)	Water quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium by determination of oxygen demand in a closed respirometer
ISO 9439 (1999)	Water quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – Carbon dioxide evolution test
ISO 9887 (1992)	Water quality – Evaluation of the aerobic biodegradability of organic compounds in an aqueous medium – Semi-continuous activated sludge method (SCAS)
ISO 9888 (1999)	Water quality – Evaluation of ultimate aerobic biodegradability of organic compounds in aqueous medium – Static test (Zahn-Wellens method)
ISO 10707 (1994)	Water quality – Evaluation in an aqueous medium of the “ultimate” aerobic biodegradability of organic compounds – Method by analysis of biochemical oxygen demand (closed bottle test)
ISO 10708 (1997)	Water quality – Evaluation in an aqueous medium of the ultimate aerobic biodegradability of organic compounds – Determination of biochemical oxygen demand in a two-phase closed bottle test
ISO 11733 (2004)	Water quality – Determination of the elimination and biodegradability of organic compounds in an aqueous medium – Activated sludge simulation test
ISO 14592-1 (2002)	Water quality – Evaluation of the aerobic biodegradability of organic compounds at low concentrations – Part 1: Shake-flask batch test with surface water or surface water/sediment suspensions
ISO 14592-2 (2002)	Water quality – Evaluation of the aerobic biodegradability of organic compounds at low concentrations – Part 2: Continuous flow river model with attached biomass
ISO 14593 (1999)	Water quality – Evaluation of the ultimate aerobic biodegradability of organic compounds in aqueous medium – Method by analysis of inorganic carbon in sealed vessels (CO ₂ headspace test)
ISO 14851 (1999)	Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium – Method by measuring the oxygen demand in a closed respirometer
ISO 14852 (1999)	Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium – Method by analysis of evolved carbon dioxide



3.1.3.1 International standards with regard to organic compounds

A brief overview of the measurement techniques is given in Table 16.

Table 16. Overview of measurement techniques of biodegradability test methods for organic compounds (ISO).

ISO method	Measurement technique	Comparable guidelines
7827	Test item (10-40 mg DOC/l) in an inoculated mineral medium is aerated at 22°C ± 2°C. Degradation is followed by DOC analysis. Degree of biodegradation is calculated by expressing the concentration of DOC removed (after correction for blank). Duration is normally 28 days or longer if necessary.	OECD 301 A
9408	Test item (at least 100 mg ThOD/l) in an inoculated mineral medium is stirred at 20°C – 25°C. Evolved CO ₂ is absorbed by soda lime or KOH or another suitable absorbent. Oxygen uptake is measured by a suitable respirometer. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD. Duration is normally 28 days or longer if necessary.	OECD 301 F ISO 14851
9439	Test item (10-40 mg organic carbon/l) in an inoculated mineral medium is aerated with CO ₂ free air at 20°C – 25°C. Degradation is followed by determining the CO ₂ produced. The produced CO ₂ is trapped in external vessels and is measured by DIC analyser or by titration after absorption in an alkaline solution. Degree of biodegradation is calculated by expressing produced CO ₂ (after correction for blank) as a percentage of ThCO ₂ . Duration is normally 28 days or longer if necessary.	OECD 301 B ISO 14852
9887	Test item (20 mg DOC/l) is exposed to high concentrations of micro-organisms (1 g/l to 4 g/l suspended solids) in an aeration (SCAS) unit at 20°C – 25°C. These conditions are highly favourable for adaptation and extensive biodegradation. At least 3 times per week the aeration is stopped, the sludge is allowed to settle (30 min) and supernatant liquor is removed. Remaining sludge is then again mixed with test compound and sewage and the cycle is repeated. Biodegradation is established by determination of DOC content of the supernatant liquor. Duration varies between 12 weeks to 26 weeks.	OECD 302 A
9888	Test item (50-400 mg DOC/l) in an inoculated (0.2 g suspended solids/l final mixture for 50 mg DOC/l – 1 g suspended solids/l final mixture for 400 mg DOC/l) mineral medium is aerated at 20°C – 25°C. These conditions with a high inoculum concentration are optimal for allowing the maximum value of biodegradation. Degree of biodegradation is calculated by expressing the concentration of DOC removed. Duration is normally 28 days.	OECD 302 B
10707	Test item (2 mg/l) in mineral medium is inoculated with relatively small number of micro-organisms and kept in closed bottles at 20°C - 25°C. The bottles are completely filled. Degradation is followed by analysis of DO. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD. Duration is 28 days.	OECD 301 D ISO 10708



ISO method	Measurement technique	Comparable guidelines
10708	Test item (100 mg ThOD/l) in mineral medium inoculated with stabilised inoculum is stirred in closed bottles, containing known volumes of medium and air, at 20°C - 25°C. Degradation is followed by analysis of DO concentration. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD. Duration is normally 28 days or longer if necessary.	
11733	Test item (10-20 mg DOC/l) is added together with an easily biodegradable organic medium to the influent of a continuously operating test system simulating an activated-sludge process (activated sludge plant = Husmann apparatus or porous pot) with a mean HRT of 6 h and a mean SRT of 6 d to 10 d at 20°C – 25°C. Effluent samples are analysed for DOC. The difference between DOC values in effluent of test and control unit compared to the influent concentration is used to determine the degree of elimination of the test compound. Duration is 12 weeks.	OECD 303A
14592-1	Test item (2 different concentration levels < 100 µg/l) is added to stirred surface water (= pelagic test) or to surface water and sediment at 100 mg/l to 1000 mg/l (= suspended sediment test) at field temperature or at a temperature of 20°C to 25°C and serves as secondary substrate (= first order biodegradation kinetics). The degradation is followed by the determination of the residual concentration of the test compound by radiotracer technique (= ¹⁴ C-labelling of the most stable part of the molecule and liquid scintillation counting) or by chemical analysis.	OECD 309
14592-2	Test item (< 200 µg/l) is added to tap water or surface water, which might be inoculated at the beginning of the water dosage, in a cascade system, each usually containing 7 trays in the form of an aquatic staircase model. Each tray is filled with glass beads, which serve as an artificial sediment to support biofilm growth. The test system simulates the conditions of a dynamic aerobic surface water system (= river). The test item serves as secondary substrate (= first order biodegradation kinetics). The difference between the inlet and outlet concentrations of the cascade is used to determine the degree of biodegradation. The test item concentration is determined by radiotracer technique (= ¹⁴ C-labelling of the most stable part of the molecule and liquid scintillation counting) or by chemical analysis. The maximum test duration is 8 weeks.	OECD 303B
14593	Test item (2-40 mg C/l) is incubated in a inoculated mineral medium in a sealed bottle with a headspace of air at 20°C - 25°C. The CO ₂ evolution is determined by measuring the IC produced in the test bottles in excess of that produced in the blank vessels. The IC is measured by (A) acidification to pH < 3 or (B) conversion of CO ₂ from the headspace to carbonate. The extent of biodegradation is expressed as a percentage of ThIC (after correction for the blank). The duration is normally 28 days or longer if necessary.	OECD 310



An overview of the different inoculum sources as prescribed by the international standards related to aerobic aqueous biodegradation of organic compounds is given in Table 17. ISO prescribes that normally no pre-exposed inoculum should be used, especially in the case of standard tests simulating biodegradation behaviour in natural environments. Depending on the purpose of a test, a pre-exposed inoculum may be used, provided this is clearly stated in the test report (% biodegradation = x % using pre-exposed inocula) and the method of pre-exposure needs to be clarified. ISO 7827, ISO 9408, ISO 9439 and ISO 10707 mention that approximately 10^3 to 10^6 colony-forming units per millilitre should be present in the final mixture. ISO 10708 refers to an inoculum, which gives between 10^4 and 10^8 active cells per millilitre. ISO 14593 recommends 10^2 to 10^5 colony forming units per millilitre in the final mixture. The prescribed amount of replicates for the different test methods is given in Table 18.

Table 17. Overview of inoculum sources of biodegradability test methods for organic compounds (ISO).

Source of inoculum	7827	9408	9439	9887	9888	10707	10708	11733	14592-1	14592-2	14593
Activated sludge from aeration tank	X	X	X	X	X		X	X			X
Waste water (influent or effluent)		X	X								X
Surface water	X	X	X			X	X		X	X	X
Secondary effluent	X					X	X	X		X	
Surface water & sediment									X		
Soil extract										X	
Mixture of sources	X	X	X			X	X			X	X

Table 18. Amount of replicates as prescribed by biodegradability test methods for organic compounds (ISO).

ISO method	Blank series	Reference series	Test series	Abiotic sterile control ⁹	Toxicity control ¹⁰
7827	2	1	2	1 (if needed)	1 (if needed)
9408	2	1	2	1 (if needed)	1 (if needed)
9439	2	1	2	1 (if needed)	1 (if needed)
9887	1	1 (optionally)	1	-	-
9888	1	1	1	1 (if needed)	-
10707	10	10	10	-	6 (if needed)
10708	3	3	3	1 (if needed)	1 (if needed)
11733	1	-	1	-	-
14592-1	1	2 (optionally)	(2 ×) ¹¹ 2	1 (optionally)	-
14592-2	-	1 (optionally)	1	-	-
14593 ¹²	> 9	> 9	> 9	> 6 (if needed)	> 6 (if needed)

⁹ Series containing test substance and sterilizing agent.

¹⁰ Series containing test substance, reference compound and inoculum.

¹¹ Two replicates for each test concentration.

¹² Number of vessels will depend on the frequency of analysis and the confidence limits required for the final extent of biodegradation. At least 5 vessels are required at the end of the test.



In order to check *the validity of the test procedure*, the most international standards prescribe that a reference compound of known biodegradability needs to be tested in parallel. ISO 9408, ISO 9439 and ISO 14593 propose aniline or sodium benzoate as reference compound, while ISO 7827, ISO 10707 and ISO 10708 propose aniline, sodium acetate or sodium benzoate. ISO 14592-1 and ISO 14592-2 only refer to aniline. No reference compound is mentioned in ISO 9887 and ISO 11733. ISO 9888 refers to diethylene glycol, ethylene glycol, sodium benzoate or aniline as suitable reference materials. The validity criteria depend on the used method (Table 19).

Table 19. Validity criteria as prescribed by biodegradability test methods for organic compounds (ISO).

ISO method	Validity criteria
7827	Difference between degradation values of replicates of test item at end < 20 % DOC removal Reference > 70 % DOC removal (after 14 days) DOC contributed by the inoculum < 10 % contributed by test compound
9408	Reference > 60 % ThOD (after 14 days) BOD blank inoculum < 60 mg O ₂ /l (after 28 days)
9439	Reference > 60 % ThCO ₂ (after 14 days) DIC at start < 5 % organic carbon of the test item CO ₂ blank inoculum < 70 mg CO ₂ /l (at end)
9887	-
9888	Reference > 70 % DOC removal (after 14 days)
10707	O ₂ uptake in blank bottles < 1.5 mg O ₂ /l (after 28 days) Residual oxygen concentration test bottles > 0.5 mg/l (any time) Difference between extremes degradation values at end < 20 % Reference > 60 % ThOD (after 14 days)
10708	Difference between extremes degradation values at end < 20 % Reference > 60 % ThOD (after 14 days) O ₂ uptake in blank bottles < 3 mg O ₂ /l per week (week 1) O ₂ uptake in blank bottles < 1 mg O ₂ /l per week (following weeks)
11733	Degree of DOC (or COD) degradation of the control units > 80% (after 2 weeks) No unusual observations
14592-1	Reference substrate needs to degrade sufficiently within the expected time interval (rate constants aniline: 0.3 d ⁻¹ – 1.7 d ⁻¹)
14592-2	Biodegradation reference substrate > 90% (after 14 days)
14593	Reference > 60 % ThIC (after 14 days) TIC in the blank controls at end ≤ 15% organic carbon added initially as test compound



3.1.3.2 International standards with regard to plastics

A brief overview of the measurements techniques is given in Table 20. The main difference with the methods for organic compounds (ISO 9408 and ISO 9439) is (1) the higher test concentration for plastic materials, (2) the longer maximum duration and (3) the possibility to use compost extract as inoculum.

Table 20. Overview of measurement techniques of biodegradability test methods for plastic materials (ISO).

ISO method	Measurement technique	Comparable guidelines
14851	Test item (100 mg/l or 170 mg ThOD/l) in an inoculated mineral medium is stirred at 20°C – 25°C. Evolved CO ₂ is absorbed in a suitable absorber. The consumption of oxygen (BOD) is determined by measuring the amount of oxygen required to maintain a constant volume of gas in the respirometer flasks or by measuring the change in volume of pressure in the flasks. Degree of biodegradation is calculated by expressing consumed oxygen (after correction for blank and correction for nitrification) as a percentage of ThOD. Maximum duration is 6 months.	OECD 301 F ISO 9408
14852	Test item (100 mg TOC/l at start) in an inoculated mineral medium is aerated with CO ₂ free air at 20°C – 25°C and degradation is followed by the determination of the CO ₂ produced. The produced CO ₂ is trapped in external vessels and is measured by DIC analyser or by titration after absorption in an alkaline solution. Degree of biodegradation is calculated by expressing produced CO ₂ (after correction for blank) as a percentage of ThCO ₂ . Maximum duration is 6 months.	OECD 301 B ISO 9439

An overview of the different inoculum sources as prescribed by ISO 14851 and ISO 14852 is given in Table 21. ISO prescribes that normally no pre-exposed inoculum should be used, especially in the case of standard tests simulating biodegradation behaviour in natural environments. Depending on the purpose of a test, a pre-exposed inoculum may also be used, provided this is clearly stated in the test report (% biodegradation = x % using pre-exposed inocula) and the method of pre-exposure needs to be clarified. Both standards recommend that the test mixture should preferably contain about 10³ - 10⁶ CFU/ml. The tests are normally executed at a constant temperature between 20°C and 25°C, but it is mentioned that higher temperatures might be appropriate when compost is used as inoculum. Higher test item concentrations (up to 2000 mg/l of organic carbon) can also be tested in both methods on condition that the optimised test medium is used. The optimized medium is highly buffered and contains more inorganic nutrients.

Table 21. Overview of inoculum sources of biodegradability test methods for plastic materials (ISO).

Source of inoculum	ISO 14851	ISO 14852
Activated sludge from aeration tank	X	X
Compost extract	X	X
Soil extract	X	X
Mixture of sources	X	X



The number of replicates in order to execute the test is identical in both standards (Table 22).

Table 22. Amount of replicates as prescribed by biodegradability test methods for plastic materials (ISO).

Method	Blank series	Reference series	Test series	Abiotic sterile control	Negative control	Toxicity control
ISO 14851	2	1	2	1 (optionally)	1 (optionally)	1 (optionally)
ISO 14852	2	1	2	1 (optionally)	1 (optionally)	1 (optionally)

In order to check *the validity of the test procedure*, ISO 14851 and ISO 14852 prescribe that a reference compound needs to be tested in parallel. These standards propose the organic compound aniline and also the biodegradable polymers microcrystalline cellulose powder, ashless cellulose filters and poly- β -hydroxybutyrate as reference materials (Figure 7). As these standards are both based on respirometric methods, they require that the degree of biodegradation of the reference material is at least 60% at the end of the test.

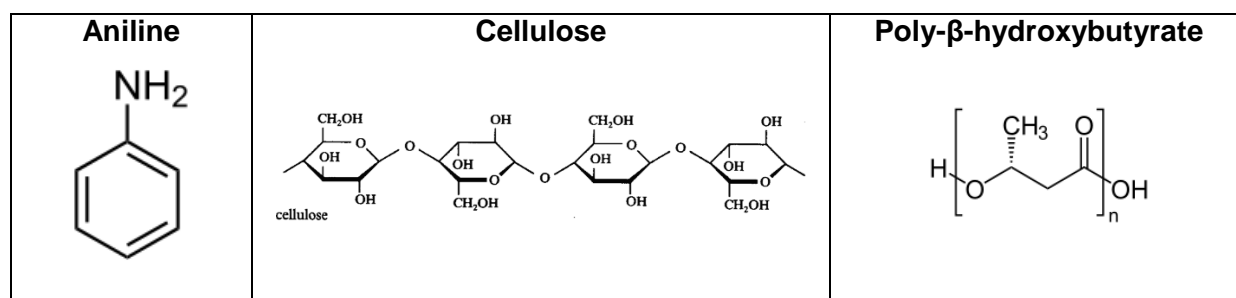


Figure 7. Structural formula of the reference compounds as described in ISO 14851 (1999) and ISO 14852 (1999).

Next to the validation criterion, which is related to the biodegradation of the reference material, also a validation criteria, which is related to the background activity of the blank is given. ISO 14851 prescribes that the BOD of the blank may not exceed 60 mg/l at the end of the test (in case of 30 mg/l dry matter), while ISO 14852 prescribes that the maximum amount of evolved carbon dioxide from the blank may not exceed 90 mg/l (in case of 30 mg/l dry matter).

ISO 14851 and ISO 14852 mention both that the determination of the BOD (inclusive the correction for nitrification) and the determination of the evolved CO_2 , respectively, are sometimes not enough in order to characterise the biodegradability of test materials with complex compositions. During a biodegradation test with a long duration the carbon in the test material is partly transformed to biomass but not biochemically oxidized. In this case, the measurements will not reach 100 % of the theoretical values even in case of complete biodegradation of the test material. In order to confirm complete biodegradability, a carbon balance can be determined. The total carbon sum is based on 5 measurements: (1) carbon evolved as CO_2 , (2) carbon produced as biomass, (3) carbon transformed into water-soluble organic metabolites, (4) carbon determined as DOC and (5) carbon remaining in the undegraded polymer material.



3.1.4 American standards

ASTM had developed standards for different product categories: (1) lubricants, (2) organic compounds and (3) plastics. No specific standards towards solvents are developed yet.

3.1.4.1 American standards with regard to lubricants

ASTM has developed 3 standard test methods for the determination of the aerobic aquatic biodegradation of lubricants and 1 standard test method for predicting the biodegradability of liquid-based lubricants using a bio-kinetic model (Table 23). Standard test methods ASTM D 5864 and ASTM D 6139 are not designed for volatile lubricants or lubricants components, while standard test method ASTM D 6731 is suitable for evaluating the biodegradation of non-volatile as well as volatile lubricants.

Table 23. Overview of the ASTM standards with regard to biodegradation in aerobic aqueous environment of lubricants.

Standard	Description
D 5864 - 11	Standard Test Method for Determining Aerobic Aquatic Biodegradation of Lubricants or Their Components
D 6139 - 11	Standard Test Method for Determining the Aerobic Aquatic Biodegradation of Lubricants or Their Components Using the Gledhill Shake Flask
D 6731 - 01	Standard Test Method for Determining the Aerobic, Aquatic Biodegradability of Lubricants or Lubricant Components in a Closed Respirometer
D 7373 - 12	Standard Test Method for Predicting Biodegradability of Lubricants Using a Bio-kinetic Model

A brief overview of the different measurements techniques is given in Table 24.

Table 24. Overview of measurement techniques of biodegradability test methods for lubricants (ASTM).

ASTM method	Measurement technique
D 5864	Produced CO ₂ is captured in Ba(OH) ₂ and amount of absorbed CO ₂ is determined by titration
D 6139	Produced CO ₂ is captured in Ba(OH) ₂ or other alkaline solution and amount of absorbed CO ₂ is determined by titration
D 6731	Oxygen consumption is determined by measuring the amount of O ₂ required to maintain a constant gas volume or by measuring the change in volume or pressure

As these methods are the only available biodegradation standard test methods for lubricants, their principles are described in detail below.



Standard test method **ASTM D 5864** is composed of (1) a CO₂ scrubbing apparatus, which consists of five 1 l plastic bottles containing 10 M NaOH, an empty Erlenmeyer flask (to prevent liquid carryover), one 1 l Erlenmeyer flask containing 0.0125 M Ba(OH)₂ and finally again an empty Erlenmeyer flask, (2) an incubation/biodegradation apparatus, which consists of a 4 l Erlenmeyer flask, and (3) a CO₂ trapping apparatus, which consists of three bottles containing 0.0125 M Ba(OH)₂ (Figure 8).

The CO₂ scrubbing apparatus produces CO₂ free air, which is used to aerate continuously the incubation apparatus. The incubation apparatus is filled with mineral medium, which contains ammonium sulphate solution, calcium chloride solution, ferric chloride solution, magnesium sulphate solution and a phosphate buffer, and 1 % activated sludge supernatant to give 30 mg/l suspended solids in the final volume of 3 l. These flasks are agitated with a magnetic stirrer. No additional carbon source is added to the blank reactors, while the reference item and the test item are added in a concentration of 10-20 mg C/l to the control reactors and the test reactors, respectively. The produced CO₂ reacts with Ba(OH)₂ and precipitates as BaCO₃ in the CO₂ trapping apparatus. The bottles of the CO₂ trapping apparatus are titrated on a regular basis. The amount of CO₂ produced is determined by titrating the remaining Ba(OH)₂ with hydrochloric acid.

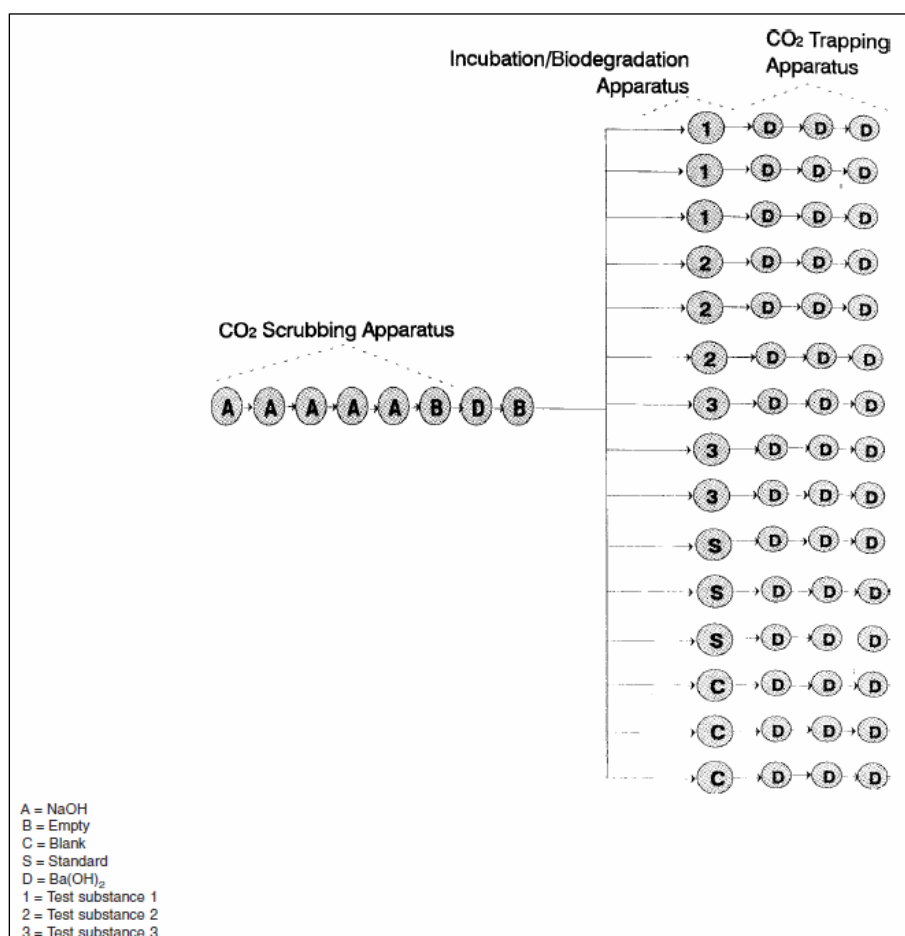


Figure 8. Schematic presentation of the aerobic aquatic biodegradation testing according to ASTM D 5864 (Source: ASTM D 5864-11).



Standard test method **ASTM D 6139** is composed of (1) a CO₂ scrubbing apparatus, which consists of a 1 l Erlenmeyer flask containing 10 M NaOH and a 1 l Erlenmeyer flask containing distilled water (optionally also a flask can be added containing 0.1 M Ba(OH)₂ to monitor a possible breakthrough of CO₂ and an empty flask to prevent liquid carryover), (2) an incubation/biodegradation apparatus (= a Gledhill-type Shake Flask Unit with a volume of 2 l), and (3) a CO₂ trapping apparatus, (conical alkaline trap unit containing 10 ml 0.1 M Ba(OH)₂) (Figure 9).

The incubation/biodegradation apparatus is filled with a mineral medium, which contains ammonium sulphate solution, calcium chloride solution, ferric chloride solution, magnesium sulphate solution, a phosphate buffer and a trace elements solution, and 1 % activated sludge supernatant to give 30 mg/l suspended solids in the final volume. This mineral medium is identical to the mineral medium of ASTM D 5864 – 11, except for the presence of the trace element solution. Before start-up of the test the aqueous mixture in the Erlenmeyer flask is aerated with CO₂ free air for at least 1 hour to purge the system of CO₂, after which the system is closed airtight. These flasks are agitated with a magnetic stirrer. No additional carbon source is added to the blank reactors, while the reference item is added to the control reactors (10-20 mg C/l) and the test item is added to the test reactors (10-20 mg C/l). The produced CO₂ reacts with Ba(OH)₂ in the conical trap and precipitates as BaCO₃. Once significant BaCO₃ is evident, Ba(OH)₂ solution is removed for titration with hydrochloric acid. Subsequently the trap is refilled with Ba(OH)₂, the inlet and outlet tubes of the flask are opened and the content of the flasks is then sparged with CO₂ free air through the inlet tube.

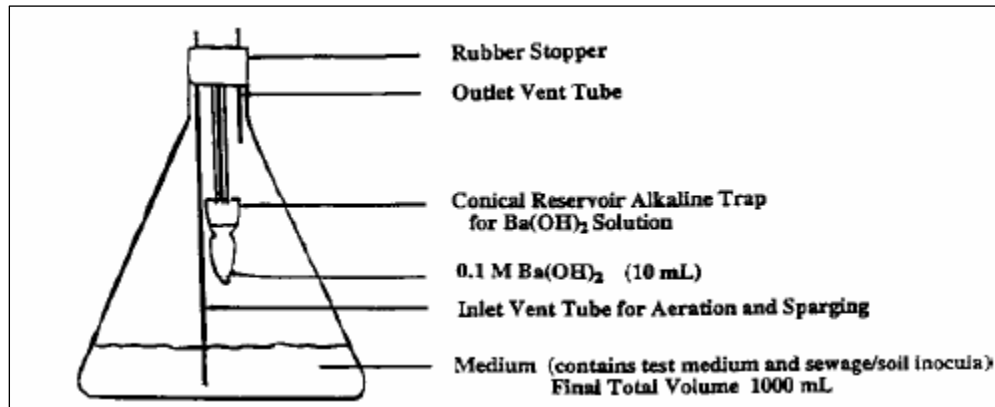


Figure 9. Presentation of the Gledhill shake flask system (Source: ASTM D 6139 - 11).

It can be concluded that the principle of methods ASTM D 5864 and ASTM D 6139 is identical (the produced CO₂ is captured in Ba(OH)₂ and the amount of CO₂ is determined by the titration of the remaining Ba(OH)₂). The main difference between these two methods is the aeration: test method ASTM D 5864 prescribes that the incubation apparatus is continuously aerated with CO₂ free air, while test method ASTM D6139 is a closed system (without continuous aeration).



Standard test method **ASTM D 6731** is composed of a closed respirometer (Figure 10), which contains a CO₂-absorber compartment. The closed respirometer is filled with a mineral medium, which contains calcium chloride solution, ferric chloride solution, magnesium sulphate solution and a phosphate buffer, and 1 % activated sludge supernatant to give 30 mg/l suspended solids in the final volume. In contrast to test methods ASTM D 5864 and ASTM D 6139 no ammonium sulphate solution (40 g/l) is added. The only source of nitrogen in the mineral medium is present in the phosphate buffer (containing 0.5 g NH₄Cl/l). Moreover, the presence of NH₄Cl in the phosphate buffers of test methods ASTM D 5864 and ASTM D 6139 is also higher (1.7 g/l). The composition of this mineral medium is identical to the mineral medium as described in the international test methods ISO 9408 (1999) and ISO 14851 (1999), which are also based on oxygen consumption. The flasks are agitated with a magnetic stirrer. No additional carbon source is added to the blank reactors, while the reference item is added to the reference reactors (50-200 mg ThOD/l) and the test item is added to the test reactors (50-200 mg ThOD/l). Alkaline solution (10 M NaOH or 10 M KOH) is added to the CO₂-absorber compartment. The produced CO₂ reacts with an absorbent solution. Consequently the total pressure in the flask decreases. The pressure drop is detected by a manometer, which produces a signal that results in the electrolytic generation of oxygen. The signal is stopped when the original pressure is re-established and the quantity of the electricity is measured. The amount of electricity used is proportional to the amount of the consumed oxygen.

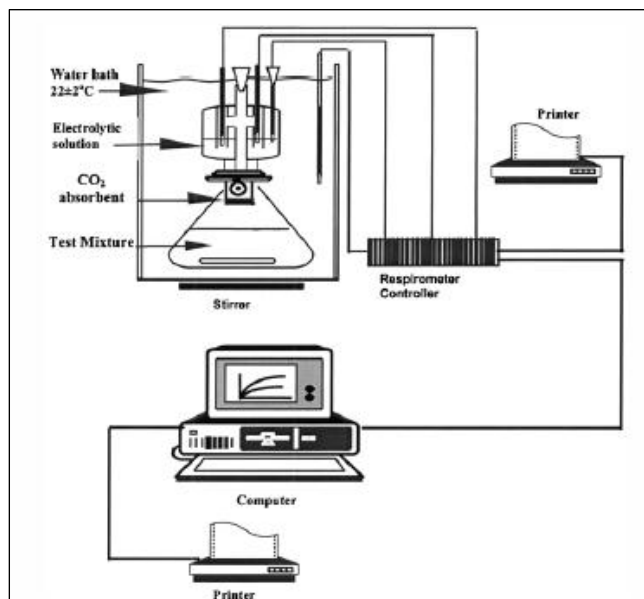


Figure 10. Principle of a closed respirometer (Source: ASTM D 6731 - 01 (2011)).



The source of inoculum as prescribed by the American test methods is given in Table 25. Pre-adaptation of the inoculum to a test material is allowed in these test methods.

Table 25. Overview of inoculum sources of biodegradability test methods for lubricants (ASTM).

Source of inoculum	D 5864	D 6139	D 6731
Wastewater-treatment plant: activated sludge	X	X	X
Secondary effluent			X
Surface water	X	X	X
Soil extract	X	X	X
Mixture of sources	X	X	X

In order to *verify the activity of the inoculum* a reference or control sample known to biodegrade needs to be tested in parallel with the test item. Test is regarded invalid if the reference does not demonstrate > 60 % of the ThCO₂ (ASTM D 5864 and ASTM D 6139) or > 60 % of the ThOD (ASTM D 6731) within 28 days. The test methods suggest using sodium benzoate or aniline for water soluble test materials and low erucic acid rapeseed oil (for example Canada oil) for water insoluble test materials.

Besides the validity criteria based on the biodegradation rate of the reference material, also a *validation criteria with regard to the blank* is given in test methods ASTM D 5864 and ASTM D 6731. ASTM D 5864 prescribes that a total CO₂ evolution in the blank at the end of the test > 75 mg CO₂ per 3 l of medium shall be considered as invalidating the test, while ASTM D6731 prescribes that a total oxygen utilization in the blank at the end of the test > 60 mg O₂/l invalidates the test. ASTM D 6139 has no validation criteria with regard to the blank series.

Moreover, also a criterion with regard to *the quantity of micro-organisms* in the inoculum is given. An overview of the required range of colony-forming units is given in Table 26.

Table 26. Amount of colony-forming units per millilitre as prescribed by biodegradability test methods for lubricants (ASTM).

ASTM method	Colony-forming units per millilitre
D 5864	$10^6 - 20 \times 10^6$
D 6139	$10^6 - 10^8$
D 6731	$10^6 - 10^7$

Test method ASTM D 5864 is run at 20 to 25°C, while methods ASTM D 6139 and ASTM D 6731 are both run at 22°C ± 2°C. Test method ASTM D 5864 needs to be executed in triplicate, while methods ASTM D 6139 and ASTM D 6731 may be executed in duplicate (although triplicates are preferred).

The minimum duration of these test methods is 28 days, but the test can be extended until the CO₂ production has reached a plateau.



The standard test method **ASTM D 7373** predicts the biodegradability of liquid-based lubricants within 1 day using a bio-kinetic model without dealing with micro-organisms. This test method is based on the determination of the content of nonaromatics, non-polar aromatics, ester fraction and polar aromatics using a glass chromatographic column packed with activated bauxite and silica gel. The different fractions are determined using specific eluents:

- Nonaromatics: n-pentane
- Non-polar aromatics: Toluene and n-pentane
- Ester fraction: Diethyl ether
- Polar aromatics: Chloroform and ethyl alcohol

The effective composition to biodegradation (ECB) is calculated only taken into account the nonaromatics and the esters. Based on the ECB the percentage of biodegradation is determined using a bio-kinetic model. According to ASTM D 7373 excellent correlation is established between the results based on the bio-kinetic model and the conventional biodegradation tests.



3.1.4.2 American standards with regard to organic compounds

An overview of the American standards with regard to aerobic biodegradation of organic compounds in aqueous aerobic environments is given in Table 27. As these guidelines are comparable to guidelines described in earlier chapters, no detailed description of the measurements techniques is given.

Table 27. Overview of the ASTM standards with regard to biodegradation in aerobic aqueous environment of organic compounds.

Standard	Description	Comparable guidelines
E1279 – 89 (2008)	Standard Test Method for Biodegradation By a Shake Flask Die Away Method	OECD 309, ISO 14592-1
E1625 – 94 (2008)	Standard Test Method for Determining Biodegradability of Organic Chemicals in Semi Continuous Activated Sludge (SCAS)	OECD 302 A, ISO 9887
E1720 – 01 (2008)	Standard Test Method for Determining Ready, Ultimate, Biodegradability of Organic Chemicals in a Sealed Vessel CO ₂ Production Test	OECD 301 B, ISO 9439
E1798 – 96 (2008)	Standard Test Method for Assessing Treatability or Biodegradability, or Both, of Organic Chemicals in Porous Pots	OECD 303 A, ISO 11733



3.1.4.3 American standards with regard to plastic materials

An overview of the American standards with regard to aerobic biodegradation of plastic materials in aqueous aerobic environments is given in Table 28. Standard test method ASTM D 6340 can be used in order to determine biodegradation in aqueous and in compost environment. Only the aqueous environment is discussed in this chapter. A brief overview of the measurements techniques of the different test methods is given in Table 29.

Table 28. Overview of the ASTM standards with regard to biodegradation in aerobic aqueous environment of plastic materials.

Standard	Description
D 5271-02	Standard Test Method for Determining the Aerobic Biodegradation of Plastic Materials in an Activated-Sludge-Wastewater-Treatment System
D 6340-98	Standard Test Methods for Determining Aerobic Biodegradation of Radiolabeled Plastic Materials in an Aqueous or Compost Environment

Table 29. Overview of measurement techniques of biodegradability test methods for plastic materials (ASTM).

Method	Measurement technique	Comparable guidelines
D 5271	Test item (at least 60 mg/l) in an inoculated mineral medium is stirred at $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Evolved CO_2 is absorbed in KOH absorber with phenolphthalein indicator. The consumption of oxygen (BOD) is determined by measuring the amount of oxygen required to maintain a constant volume of gas in the respirometer flasks. Moreover, also the soluble-organic-carbon (at start and at end), nitrite/nitrate (at start and at end) and insoluble plastic (at end) are determined in order to determine the biodegradation percentage. Correction needs to be made for oxygen uptake due to nitrification. Maximum duration is 6 months.	OECD 301 F ISO 9408 ISO 14851
D 6340	A radiolabeled plastic (50-100 mg per 80 ml medium) in an inoculated limited basal media (BAM broth minus glucose) is aerated at $58^{\circ}\text{C} \pm 5^{\circ}\text{C}$. The produced gasses pass through an acid trap and consequently through a CO_2 absorption column containing cooled methoxyethyl amine. The $^{14}\text{CO}_2$ absorbed in the methoxyethyl amine is determined in a liquid scintillation counter, which measures the beta radiation of ^{14}C .	

An overview of the different inoculum sources is given in Table 30. ASTM D 5271 mentions that adaptation of the biomass may be desirable to provide increased reproducibility.

Table 30. Overview of the different inoculum sources as prescribed by ASTM.

Source of inoculum	D 5271	D 6340
Activated sludge from aeration tank	X	X
Soil extract	X	
Compost extract	X	X
Other environmental sources		X
Waste water		X
Mixture of sources	X	X



The prescribed amount of the different series is given in Table 31.

Table 31. Amount of replicates as prescribed by biodegradability test methods for plastic materials (ASTM).

ASTM method	Blank series	Reference series	Test series	Negative control
D 5271	3	3	3	3 (optional)
D 6340			1	

In order to check *the activity of the inoculum*, ASTM D 5271 mentions analytical-grade cellulose and starch as suitable reference material. The validation criteria depend on the used method (Table 32). No reference material, nor validation criteria is mentioned in ASTM D 6340.

Table 32. Validity criteria as prescribed by biodegradability test methods for plastic materials (ASTM).

ASTM method	Validity criteria
D 5271	Reference > 60 % ThOD (at end) BOD blank inoculum < 60 mg O ₂ /l (at end)
D 6340	-



3.2 Toxicity

Aquatic toxicity refers to the intrinsic property of a substance to be detrimental to an organism in short-term and/or long-term exposure to that substance. In general, it is assumed that the aquatic toxicity is mainly related to waterborne exposure of a substance and expressed as external concentration of that substance in test water, but there may be cases where food uptake is the predominant route of exposure (i.e. for very hydrophobic or very sorptive substances). It is believed that substances dissolved in water and taken up by organisms may accumulate to a certain internal concentration, which may then cause adverse effects. Therefore factors that influence bioconcentration (molecular weight, water solubility and $\log K_{ow}$) influence also toxicity to aquatic species.

There exist 2 types of aquatic toxicity tests: (1) acute toxicity tests and (2) chronic toxicity tests (ECHA (2012)).

Acute toxicity related to waterborne exposure is generally expressed in terms of a concentration which is lethal to 50 % of the test organisms (lethal concentration, LC_{50}), causes a measurable adverse effect to 50 % of the test organisms (e.g. immobilization of daphnids), or leads to a 50 % reduction in test (treated) organism responses from control (untreated) organism responses (e.g. growth rate in algae) following an exposure in the range of hours to days. This is expressed as effective concentration (EC_{50}).

Chronic toxicity related to waterborne exposure refers to the potential or actual properties of a substance to cause adverse effects to aquatic organisms during exposures which are determined in relation to the life-cycle of the organism. Such chronic effects usually include a range of sublethal endpoints and are generally expressed in terms of NOEC (No Observed Effect Concentration), LOEC (Lowest Observed Effect Concentration), EC_x or MATC (Maximal Acceptable Toxicant Concentration). Observable endpoints in chronic studies typically include survival, growth and/or reproduction.

There exist different types of information relevant for assessing aquatic toxicity. This includes “in vivo” testing methods and non-testing methods (QSAR, etc.). Currently no EU / OECD guidelines for “in vitro” tests relevant to aquatic toxicity are available.

The OECD guidelines for aquatic toxicity testing comprise internationally agreed testing methods for environmental effects. Tests undertaken following the OECD guidelines are useful for both risk assessment and classification purposes. The obtained data are covered by the principle of mutual acceptance of data. Therefore, the OECD guidelines related to aquatic toxicity are described in detail in chapter 3.2.2, while only an overview is given of the other existing standard test methods (international standards and American standards). Chapter 3.2.1 describes useful preparation techniques in order to evaluate aquatic toxicity of poorly water soluble and volatile substances.



3.2.1 Preparation of the test items

ASTM has developed a specific standard with regard to the sample preparation and the results interpretation of aquatic toxicity tests on lubricants: *ASTM D 6081 – 98 (Reapproved 2009) “Standard Practice for Aquatic Toxicity Testing of Lubricants: Sample Preparation and Results Interpretation”*. As most lubricants and lubricant components are mixtures of chemical compounds with varying and usually poor solubility in water, they are difficult to evaluate in toxicity tests. Consequently the toxicity of mixtures of poorly soluble components cannot be expressed in terms of lethal concentration, effect concentration or inhibition concentration because the mixtures may not be completely soluble at treat levels that lead to toxic effects. This standard practice prescribes that toxicity results of poorly soluble materials should better be expressed as loading rate.

The lubricants or lubricant components should not be added directly to aquatic systems for toxicity testing because the addition procedure will affect the results of the toxicity tests. Therefore ASTM D 6081 – 98 describes 3 different methods for material preparation of lubricants or lubricant components: (1) preparation of Water-Accommodated Fraction (WAF), (2) preparation of Water-Soluble Fraction (WSF) and (3) preparation of a mechanical dispersion. The disadvantage of last technique is that it cannot be used for poorly swimming organisms (due to the fact that this technique generates turbulence).

WAF refers to components dissolved in the water phase or entrained as stable droplets in the water phase, while WSF only refers to components dissolved in the water phase. The WAF is prepared by adding the test item in the desired nominal exposure load to water. The content of the vessel is stirred (vortex depth: 10 – 35 % of test solution height) for 20 to 24 hours. This is followed by a subsequent settling period of 1 to 4 hours. After the settling period the aqueous solution is decanted (= WAF). The WSF is prepared by the filtration of the WAF through a 0.45 µm cellulose-acetate filter or by centrifugation of the WAF to remove the undissolved material.

The mechanical dispersion technique is based on the presence of a motor-driven propeller assembly to move the test material and the dilution water in the test vessel during the study. A schematic presentation of this technique is given in Figure 11.

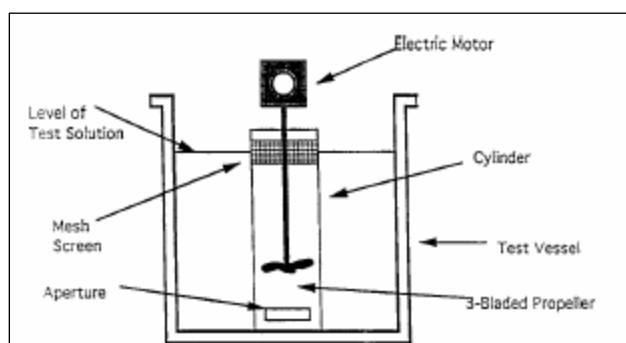


Figure 11. Schematic presentation of the mechanical dispersion technique (Source: ASTM D 6081).



Also **OECD** has developed a guidance document on aquatic toxicity testing of difficult substances and mixtures (Guidance document on aquatic toxicity testing of difficult substances and mixtures). When the concentration of a test substance declines significantly (> 20 %) during a test due to physical, chemical or biological processes, modifications to media preparation and exposure systems can be required. When no data are available in order to identify the process responsible for the decline, a preliminary study is required in order to assess the stability of the test substance. The goal of a preliminary study is to determine if a substance is stable, volatile, sorbed onto glass, photodegraded, hydrolytically unstable, oxidizable or biodegradable. The OECD guidance documents gives specific recommendations with regard to different categories of difficult substances and mixtures, but in the scope of this study only poorly water-soluble substances, volatile substances, biodegradable substances, hydrophobic substances and multi-component substances (= complex mix of individual substances with different solubility and physical-chemical properties) are described in detail.

For poorly water-soluble substances 4 methods of test media preparation are described: (1) direct addition, (2) water-miscible solvents (acetone, ethanol, methanol, tertiary-butyl alcohol, etc.), (3) generator systems (chemically inert matrix which has been coated with the substance) and (4) dispersions and emulsions.

Volatile substances can be lost by evaporation to the atmosphere. Losses of volatile substances during preparation and exposure can be minimised using relatively straightforward modifications to procedures. The guideline mentions that the vessels should be sealed during preparation and the headspace should be kept to a minimum if possible. For algal tests a sealed exposure system will result in culture growth being limited by CO₂ depletion and increasing pH. Consequently it is necessary to perform the test in a CO₂ enriched headspace or the test can be executed in a system without headspace in which gaseous CO₂ is replaced with NaHCO₃ and the pH is adjusted with HCl.

Readily biodegradable substances are likely to be degraded in aquatic test systems once competent bacterial populations become established. The maintenance of exposure concentrations is consequently dependent upon preventing the development of significant microbial populations. Therefore a strict test vessel hygiene will decay and limit, but not prevent the development of bacteria. Moreover, a flow-through exposure regime with sufficient volume renewal and a high concentration stock solution maintained under nitrogen has been shown to prevent aerobic biodegradation, minimise the concentration of breakdown products and maintain exposure concentration of the parent substance.

Hydrophobic substances ($\log K_{ow} > 4$ or BCF > 500) can be partitioned into or onto test organism biomass and onto food or other organic detritus in the test system. In order to maintain the exposure concentrations (1) the ratio test organism biomass to test medium volume can be reduced, (2) the excess food and detritus can be removed, (3) a semi-static renewal or flow-through exposure regime can be preferred, (4) the dissolved total organic carbon concentrations need to be maintained at or below 2 mg/l, (5) feeding can be executed a few hours before test medium renewal and (6) the system surfaces can be saturated with the compound.



The toxicity of multi-components substances, which are only partly soluble in water, can be determined by preparing water-accommodated fractions (WAF's).

ISO has developed a comparable document with regard to poorly soluble materials, volatile compounds, metals and wastewater, but only related to algal growth inhibition tests: *ISO 14442 (2006) "Water quality – Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water"*.

ISO 14442 prescribes that poorly soluble mixtures of organic substances (= homogenous aggregates of a number of compounds with different physic-chemical and/or chemical properties, which cannot be easily separated into their component parts by physical means and formulated products) should be tested by preparing the water-accommodated fractions (WAF's) by stirring and phase separation. Consequently, the results need to be expressed as loading rates instead of the usual concentrations. The preparation of the WAF is identical to the preparation method as prescribed by ASTM D 6081.

According to ISO 14442 volatile substances ($H > 1 \text{ Pa}\cdot\text{m}^3/\text{mol}$) should be tested in closed systems. However, as optimum algal growth and the maintenance of a stable pH require both a sufficient supply of CO_2 , the headspace needs to be enriched with CO_2 or the dissolved CO_2 needs to be increased by the addition of NaHCO_3 .



3.2.2 Test methods

3.2.2.1 OECD guidelines

An overview of the guidelines with regard to aquatic toxicity as developed by OECD is given in Table 33. These test methods are all designed to evaluate the aquatic toxicity of chemicals. Different types of freshwater aquatic species can be evaluated: (1) algae, (2) aquatic plants, (3) invertebrates and (4) fish. OECD 229 is the only guideline, which is developed in order to test potential endocrine disrupting chemicals.

A short description of the test method of these aquatic toxicity tests is given in Table 34. The validation criteria of the test methods is given in Table 35.

Table 33. Overview of the OECD guidelines with regard to freshwater aquatic toxicity.

Guideline	Adopted	Description
OECD 201	Mar-23-2006	Freshwater Alga and Cyanobacteria, Growth Inhibition Test
OECD 202	Apr-13-2004	<i>Daphnia</i> sp., Acute Immobilisation Test
OECD 203	Jul-17-1992	Fish, Acute Toxicity Test
OECD 204	Apr-04-1984	Fish, Prolonged Toxicity Test: 14-day Study
OECD 209	Jul-22-2010	Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)
OECD 210	Jul-17-1992	Fish, Early-life Stage Toxicity Test
OECD 211	Oct-02-2012	<i>Daphnia magna</i> Reproduction Test
OECD 212	Sep-21-1998	Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages
OECD 215	Jan-21-2000	Fish, Juvenile Growth Test
OECD 221	Mar-23-2006	<i>Lemna</i> sp. Growth Inhibition Test
OECD 229	Oct-02-2012	Fish Short Term Reproduction Assay



Table 34. Description of the OECD test methods for aquatic toxicity tests.

Guideline	Description
OECD 201	Effect of a substance on the growth of freshwater microalgae and/or cyanobacteria is evaluated over a period of normally 72 hours at a temperature of 21°C to 24°C. The system response is the reduction of growth (= algal biomass as a function of time) in a series of algal cultures exposed to various test concentrations. The concentration bringing about a specified x % inhibition of growth rate is determined and expressed as the E_rC_x .
OECD 202	Effect of a substance on the immobilization of daphnids with an age < 24 hours (at least 20 daphnids per test series) is evaluated over a period of 48 hours at a temperature within the range of 18°C and 22°C. Immobilization is recorded after 24 hours and after 48 hours. The results are analysed in order to calculate the EC_{50} .
OECD 203	Effect of a substance on the survival of fish (at least 7 fish per test series) is evaluated over a period of 96 hours in a static, a semi-static or a flow-through procedure at a temperature, which is adapted to the used fish species. Mortalities and other observations are recorded at 24, 48, 72 and 96 hours. The concentration, which kills 50 % of the fish (LC_{50}) is determined when possible.
OECD 204	Effect of a substance on the survival of fish (at least 10 fish per test series) is evaluated over a period of at least 14 days in a semi-static or a flow-through procedure at a temperature, which is adapted to the used fish species. Mortalities and other observations are recorded minimum three times per week. Moreover, fish should be measured and weighted before the start-up and at the termination of the test. Threshold levels of lethal and other observed effects and NOEC are determined.
OECD 209	Effect of a substance on the respiration rate of activated sludge fed with synthetic sewage is determined after a contact time of 3 hours at 20°C \pm 2°C. Respiration rate is determined by measuring the concentration of dissolved oxygen. Three different oxygen uptakes may be determined: total, heterotrophic only and that due to nitrification. The EC_x and/or the NOEC are determined.
OECD 210	Define the lethal and sub-lethal effects of a test substance on the early-life stages of fish (at least 60 fertilised eggs, divided equally between at least 2 replicate test chambers) in a semi-static or a flow-through procedure at a temperature, which is adapted to the used fish species. The test is continued at least until all the control fish are pre-feeding. The LOEC and the NOEC are determined.
OECD 211	Effect of a substance on the reproductive output of young female <i>Daphnia magna</i> with an age < 24 hours is evaluated over a period of 21 days at a temperature within the range of 18°C and 22°C. The test can be executed in a semi-static procedure (10 animals individually held per concentration) or a flow-through procedure (40 animals divided into 4 groups of 10 animals at each test concentration). At the end of the test the total amount of living offspring produced is assessed. The toxic effect of the test substance on reproductive output is expressed as EC_x or alternatively as NOEC/LOEC value.



Guideline	Description
OECD 212	Define the lethal and to a limited extent sub-lethal effects of a test substance on fish embryo and sac-fry stages (at least 30 fertilised eggs, divided equally between at least 3 replicate test chambers) in a semi-static or a flow-through procedure at a temperature, which is adapted to the used fish species. The test is terminated just before the yolk-sac of any larvae in any of the test chambers has been completely absorbed or before mortalities by starvation start in the controls. The LOEC and the NOEC are determined.
OECD 215	Effect of a substance in a range of at least 5 sublethal concentrations on the growth of juvenile fish is evaluated over a period of 28 days in a semi-static or a flow-through procedure at a temperature, which is adapted to the used fish species. The juvenile fish are weighted at start, optionally after 14 days and at the end of the test. Effects on growth rates are analysed using a regression model in order to estimate the concentration that would cause a x % variation in growth rate (EC_x). In addition LOEC and NOEC can be determined.
OECD 221	Effect of a substance in a range of at least 5 test concentrations on the vegetative growth of freshwater aquatic plants of the genus <i>Lemna</i> (duckweed) is evaluated over a period of 7 days in a static, a semi-static or a flow-through procedure at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Frond number is the primary measurement variable. At least one other measurement variable (total frond area, dry weight of fresh weight) is also measured. Concentrations bringing about a specified x % inhibition of growth (= average specific growth rate) and a x % inhibition of yield are determined and expressed as the E_rC_x and E_yC_x , respectively. In addition LOEC and NOEC can be determined.
OECD 229	Detect endocrine active substances by exposing sexually mature male and spawning female fish (zebrafish, fathead minnow or Japanese medaka) to three chemical exposure concentrations and a water control during 21 days. The amount of replicates and the amount of fish per replicate are in function of the fish species. Quantitative fecundity is monitored daily, while at the end of the test secondary sex characteristics are measured (only for fathead minnow or Japanese medaka), vitellogenin is determined and gonads are fixed for potential histopathological evaluation.

Table 35. Validity criteria of the OECD test methods for aquatic toxicity tests.

Guideline	Validity criteria
OECD 201	Biomass control cultures: exponential increase \geq factor 16 within 72 hours Mean coefficient of variation for section-by-section growth rates in control cultures < 35 % Coefficient of variation of average growth rates in replicate control cultures < 7 % (<i>Pseudokirchneriella subcapitata</i> & <i>Desmodesmus subspicatus</i>) or < 10 % (other species)
OECD 202	Immobilization in the control series < 10 % after 48 hours Dissolved oxygen concentration at the end of the test \geq 3 mg/l
OECD 203	Mortality in the control series < 10 % (at end) Constant conditions should be maintained Dissolved oxygen concentration > 60 % of air saturation value (complete test) Evidence that the concentration of the substance is maintained (at least 80% of nominal concentration)



Guideline	Validity criteria
OECD 204	Mortality in the control series < 10 % (at end) Dissolved oxygen concentration > 60 % of air saturation value (complete test) Evidence that the concentration of the substance is maintained (at least 80% of nominal concentration)
OECD 209	Oxygen uptake rate of blank controls > 20 mg O ₂ / g activated sludge (dry weight of suspended solids) in an hour Coefficient of variation of O ₂ uptake rate in control replicates < 30% at the end Range EC ₅₀ 3,5-DCP: 2 – 25 mg/L (total respiration), 5 – 40 mg/L (heterotrophic respiration) & 0.1 – 10 mg/L (nitrification respiration) Range EC ₅₀ copper (II) sulphate pentahydrate: 53 – 155 mg/L (total respiration)
OECD 210	Overall survival > limit values (which are defined per species) Dissolved oxygen concentration > 60 % of air saturation value (complete test) Variation water temperature < 1.5°C between test chambers or successive days Evidence that the concentration of the substance is maintained (within ± 20% of the mean measured values)
OECD 211	Mortality of the parent animals < 20 % (at end) Mean number of living offspring produced per surviving parent animal ≥ 60
OECD 212	Overall survival > limit values (which are defined per species) Dissolved oxygen concentration > 60 % of air saturation value (complete test) Variation water temperature < 1.5°C between test chambers or successive days
OECD 215	Mortality in the control series < 10 % (at end) Mean weight of fish in the control(s) must have increased enough to permit the detection of the minimum variation of growth rate considered as significant. Dissolved oxygen concentration > 60 % of air saturation value (complete test) Variation water temperature < 1°C between test chambers and within a range of 2°C within the temperature ranges specified for the test species
OECD 221	Doubling time of frond number in the control < 2.5 days (60 hours)
OECD 229	Mortality in the controls < 10% (at end) Dissolved oxygen concentration > 60 % of air saturation value (complete test) Variation water temperature < 1.5°C between test chambers and within a range of 2°C within the temperature ranges specified for the test species Evidence that the concentration of the substance is maintained (within ± 20% of the mean measured values) Evidence that fish are actively spawning in all replicates prior to initiating chemical exposure



3.2.2.2 International standards

An overview of the international standards with regard to freshwater aquatic toxicity is given in Table 36. It is noticed that in addition to *Daphnia* sp., also chronic toxicity tests were developed towards *Ceriodaphnia dubia* (water flea occurring in freshwater lakes, ponds and marshes) and towards *Brachionus calyciflorus* (planktonic rotifer occurring in freshwater).

Table 36. Overview of the international standards with regard to freshwater aquatic toxicity.

ISO standard	Description
6341 (2012)	Water quality – Determination of the inhibition of the mobility of <i>Daphnia magna</i> Straus (Cladocera, Crustacea) – Acute toxicity test
7346-1 (1996)	Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [<i>Brachydanio rerio</i> Hamilton-Buchanan (Teleostei, Cyprinidae)] – Part 1: Static method
7346-2 (1996)	Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [<i>Brachydanio rerio</i> Hamilton-Buchanan (Teleostei, Cyprinidae)] – Part 1: Semi-static method
7346-3 (1996)	Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [<i>Brachydanio rerio</i> Hamilton-Buchanan (Teleostei, Cyprinidae)] – Part 1: Flow-through method
8192 (2007)	Water quality – Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation
8692 (2012)	Water quality – Fresh water algal growth inhibition test with unicellular green algae
10229 (1994)	Water quality – Determination of the prolonged toxicity of substances to freshwater fish – Method for evaluation the effects of substances on the growth rate of rainbow trout (<i>Oncorhynchus mykiss</i> Walbaum (Teleostei, Salmonidae))
10706 (2000)	Water quality – Determination of long term toxicity of substances to <i>Daphnia magna</i> Straus (Cladocera, Crustacea)
12890 (1999)	Water quality – Determination of toxicity to embryos and larvae of freshwater fish – Semi-static method
20665 (2008)	Water quality – Determination of chronic toxicity to <i>Ceriodaphnia dubia</i>
20666 (2008)	Water quality – Determination of chronic toxicity to <i>Brachionus calyciflorus</i> in 48 h



3.2.2.3 American standards

An overview of the American standards with regard to aquatic toxicity is given in Table 37. In addition to the species tested in the OECD guidelines also standards towards other species were developed (amphibians, Rotifer Brachionus, freshwater mussels, etc.).

Table 37. Overview of the American standards with regard to freshwater aquatic toxicity.

ASTM standard	Description
E 729 – 96 (2007)	Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates and Amphibians
E 1193 – 97 (2004)	Standard Guide for Conducting Daphnia magna Life Cycle Toxicity Tests
E 1218 – 04e1	Standard Guide for Conducting Static Toxicity Tests with Microalgae
E 1241 – 05	Standard Guide for Conducting Early Life Stage Toxicity Tests with Fishes
E 1366 – 11	Standard Practice for Standardized Aquatic Microcosms: Fresh Water
E 1415 – 91 (2004)e1	Standard Guide for Conducting Static Toxicity Tests with Lemna gibba G3
E 1440 – 91 (2004)	Standard Guide for Acute Toxicity Test with the Rotifer Brachionus
E 1604 – 94 (2007)	Standard Guide for Behavioral Testing in Aquatic Toxicology
E 1706 – 05 (2010)	Standard Test Method for Measuring the Toxicity of Sediment Associated Contaminants with Freshwater Invertebrates
E 1711 – 95 (2008)	Standard Guide for Measurement of Behaviour During Fish Toxicity Tests
E 1850 – 04	Standard Guide for Selection of Resident Species as Test Organisms for Aquatic and Sediment Toxicity Tests
E 2455 – 06	Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels
E 2591 – 07	Standard Guide for Conducting Whole Sediment Toxicity Tests with Amphibians



3.3 Standard specifications

3.3.1 European specifications

Currently, no European specification is developed yet defining the criteria for bio-lubricants and bio-solvents, but CEN has already developed a technical report with recommendations (no specifications) for terminology and characterisation of bio-lubricants and bio-based lubricants: *CEN/TR 16227 (2011) "Liquid petroleum products – Bio-lubricants – Recommendation for terminology and characterisation of bio-lubricants and bio-based lubricants"*. This technical report recommends that the claim "bio" should refer to an international or at least European agreed standard. In order to avoid market hindering confusion, following minimum requirements are recommended in this technical report for bio-lubricants:

- Minimum content of renewable raw material: 25 %
 - Method: ASTM D 6866 or equivalent CEN version (to be developed)
- Minimum biodegradation percentage: 60 % (oils) or 50 % (lubricating greases)
 - Method: OECD 301 B, C, D or F or adequate ISO or EN standards
- Not labelled as "Dangerous to the environment" (Symbol N)
 - EC₅₀/LC₅₀/IC₅₀ fully formulated product > 100 mg/l
 - Method: OECD 201/202/203
- Minimum performance criteria
- Any lubricant according to the present criteria of the EU Ecolabel for Lubricants is per definition a bio-lubricant

The technical report also gives an overview of the biodegradation and ecotoxicity test methods which are of importance for lubricants:

- Biodegradation
 - OECD 301 B, C, D & F
 - ISO 10708
 - EN ISO 14593
- Ecotoxicity
 - Bacteria test: EN ISO 8192
 - Algae toxicity test: OECD 201
 - Daphnia test: OECD 202
 - Fish toxicity: OECD 203
 - Fish toxicity: OECD 204 (incorporated in "Blue Angel" ecolabel)
 - Plant growth method: OECD 208 (incorporated in "Blue Angel" ecolabel)

The technical report mentions that also the EC Dangerous Preparations Directive (DPD) needs to be taken into account. The DPD identifies and labels dangerous formulations in finished products. Based on the amount of Dangerous for the Environment classified components, the final lubricant might be classified as "Dangerous for the Environment" and might carry the "dead fish/dead tree" hazard symbol. The health and environmental hazards



of a preparation can be evaluated by either a conventional method (with limits for single components) or by testing the preparation using standard testing guidelines.

In order to test the preparation, all test species (algae: OECD 201, daphnia: OECD 202 and fish: OECD 203) need to be tested and the IC/EC/LC₅₀ needs to be > 100 mg/l for each trophic level. When this testing was successful, a preparation is not related to a symbol, even if the calculation scheme would demand labelling. Only if a preparation contains more than the specified amount of a component, which is classified as a carcinogen, mutagen or reproductive toxicant, the conventional test method (with limits for single components) shall be applied.

For plastic material a specification is developed towards disposability of plastics in waste water treatment systems: *EN 14987 (2006) "Plastics – Evaluation of disposability in waste water treatment plant – Test scheme for final acceptance and specifications"*. This test scheme specifies test methods and criteria which are applied in order to verify if a solid plastic material can be considered as disposable in waste water treatment systems. Materials which are shown to be in compliance with this standard can be eventually disposed of in municipal or industrial waste water treatment plants, through the sewage.

A distinction is made between cold and hot "water soluble/dispersible plastics". The hot water soluble/dispersible plastics can only be disposed of through the sewage after exposition to hot water. Moreover, also a distinction is made between soluble and dispersible plastics. The dispersible plastics are not suitable for application where the final solution shall pass through small diameter pipes or orifices (e.g. laundry bags for washing machines). An overview of the specific requirements is given in Table 38.

Table 38. Overview of the requirements for plastics disposable in waste water treatment systems as prescribed by EN 14987 (2006).

Parameter	Requirement as prescribed by EN 14987 (2006)
Biodegradation	Minimum mineralization degree of 90 % (absolute) or 90 % of the mineralization degree reached in the same time by a reference material (soluble starch or microcrystalline cellulose), tested in parallel (relative), within 56 days. (EN ISO 14851 / EN ISO 14852 at 20°C – 25°C & soil or compost extracts are not allowed)
Water dispersibility or water solubility	Plastic material must be water dispersible (dispersible fraction D ≥ 0.9 after dissolution in cold or hot water) or water soluble (soluble fraction S ≥ 0.9 after dissolution in cold or hot water)



3.3.2 International specifications

There exists an international standard, which gives specifications for environmentally acceptable hydraulic fluids (HE): *ISO 15380 (2011) "Lubricants, industrial oils and related products (class L) – Family H (Hydraulic systems) – Specifications for categories HETG, HEPG, HEES and HEPR"*. The specification is related towards ready biodegradability (in aerobic aqueous environment) and (aquatic) ecotoxicity. Only criteria are given for 4 categories: (1) HETG (triglycerides), (2) HEPG (polyglycols), (3) HEES (synthetic esters) and (4) HEPR (polyalphaolefins and related hydrocarbons). The hydraulic fluids, which meet the specifications of this standard are designed to minimize the impact upon the environment in the event of a leak or a spill. An overview of the specifications is given in Table 39.

Table 39. Overview of the requirements for environmentally acceptable hydraulic fluids as prescribed by ISO 15380 (2011).

Parameter	Requirement as prescribed by ISO 15380 (2011)
Biodegradation	Min. 60 % (ISO 14593 or ISO 9439)
Toxicity	
Acute fish toxicity, 96 h, LC ₅₀	Min. 100 mg/l (ISO 7346-2)
Acute Daphnia toxicity, 48 h, EC ₅₀	Min. 100 mg/l (ISO 6341)
Bacterial inhibition, 3 h, EC ₅₀	Min. 100 mg/l (ISO 8192)



3.4 Labelling

The objective of ecolabelling is to provide clear information to customers to allow them to select products that are the least harmful to the environment. This should stimulate environmental concern in product development. These labelling systems have defined and established methods to measure the properties of a substance that would qualify it as being environmentally acceptable.

3.4.1 Der Blaue Engel / Blue Angel

The first national labelling scheme for lubricants was the German Blue Angel label, developed in 1988 (Figure 12). This label indicates that the impact of the product on the environment is lower when compared to impact of a comparable product. Three biodegradable lubricant categories can be certified under the Blue Angel Eco-label. An overview of these categories is given in Table 40.



Figure 12. Logo of Der Blaue Engel ecolabel.

Table 40. Overview of the lubricant categories as prescribed by the Blue Angel labelling system.

Designation	
RAL-UZ 48	Readily Biodegradable Chain Lubricants for Power Saws
RAL-UZ 64	Readily Biodegradable Lubricants & Forming oils
RAL-UZ 79	Readily Biodegradable Hydraulic Fluids

The Blue Angel eco-labels are mainly focused on biodegradability, low toxicity to aquatic organisms, non-bioaccumulative, no dangerous components (such as carcinogens or toxic substances) and technical specifications. However, no criteria with regard to bio-based content are mentioned. Consequently, lubricants comprised completely of petroleum-sourced components can receive Blue Angel certification.

A significant amount of product are already certified according to the Blue Angel label:

- RAL-UZ 48: > 70 vendors & > 90 products
- RAL-UZ 64: > 25 vendors & > 50 products
- RAL-UZ 79: > 20 vendors & > 60 products



The biodegradability and eco-toxicity requirements as described in the Blue Angel ecolabel are briefly described below.

- Each basic substance (= ingredient in a concentration > 5 %) need to be biodegradable by at least 70 % (OECD 301 B, C, D & F, ISO 14593 or ISO 10708) and if it is suspected that basic substances have a toxicity < 100 mg/l following ecotoxicity tests must be conducted: OECD 202, 203, 201, 202, 204, 208, 209, DIN 38418-6.
- The content of non-biodegradable or only potentially biodegradable (OECD 302 B or C: ≥ 20 % biodegradation) ingredients must not exceed 5 weight % and there shall not be ecotoxicological doubts. Ecotoxicological doubts exist if one of following items is fulfilled:
 - Highly toxic to aquatic organisms
 - Acute: $EC_{50}/LC_{50} \leq 1$ mg/l OR
 - Chronic: $NOEC \leq 0.01$ mg/l
 - Negative impact on aquatic organisms and bioaccumulative potential
 - Acute: $EC_{50}/LC_{50} \leq 100$ mg/l OR
 - Chronic: $NOEC \leq 1$ mg/l
 - $\log P_{ow} \geq 3.0$ OR
 - $BCF > 100$ OR
 - Surface active
 - Toxic effect on vascular plants
 - $EC_{50} \leq 100$ mg/kg
 - Inhibitory to bacterial growth
 - $IC_{50} < 100$ mg/l
- If additives are non-biodegradable polymers, following items need to be fulfilled:
 - Proof of immobility
 - Water solubility < 1 mg/l
 - Components with molecular weight ≤ 1000 g/mol < 1 %
 - No ecotoxicological doubts

3.4.2 Swedish labelling scheme

Also Sweden has developed a national labelling system for lubricants. Two standards were developed:

- SS 155434: Hydraulic fluids – Requirements and test methods
- SS 155470: Lubricating grease – Requirements and test methods

The evaluation of the lubricant involves testing for biodegradability, aquatic toxicity and sensitizing properties of a lubricant formulation and its components. Depending upon class also requirements with regard to renewable resources content are defined.



3.4.3 Nordic Swan

The first international labelling program for environmentally acceptable lubricants was the Nordic Swan program (Figure 13). The Nordic Swan is the official Ecolabel for the Nordic Countries (Sweden, Norway, Finland, Iceland and Denmark). The secretariats in the participating countries are responsible for implementing the scheme on national level. It imposes stringent environmental and climate criteria towards 63 product groups. The objective of Nordic Ecolabelling is to offer consumers guidance on the selection of products within a particular group which are considered to have the least potential adverse impact on health and the environment during their lifecycle.



Figure 13. Logo of the Nordic Swan Ecolabel.

In 2000 the Nordic Swan had developed a criteria document for lubricating oils. However, due to the fact that there was no sufficient interest in this product category, the category of lubricants has been cancelled on 31 January 2012. The low interest is probably caused by the requirements for renewability, which are the highest of all the labelling programs.

The criteria of the withdrawn criteria document for ecolabelling of lubricants are described below in order to illustrate why this labelling program was too stringent.

Separate requirements were imposed to chain oil, mould oil, hydraulic oil, 2-stroke oil, lubricating grease, metal cutting fluid and transmission-/gear fluid. The products must not present an undue hazard to environment or health, must conform technical performance criteria and must have a high content of renewable resources. The requirements were set with the aim that maximum 1/3 of the products on the Nordic market should be able to fulfil the criteria.

For chain oils, mould oils, hydraulic oils, 2-stroke oils and lubricating greases requirements were imposed towards the minimum quantity of renewable raw materials (min. 85 % for chain oils and mould oils, min. 65 % for hydraulic oils and lubricating greases and min. 50 % for 2-stroke oils) and strict environmental requirements are imposed to the base oil and the additives used.

For metal cutting fluids and transmission-/gear fluids it was possible to choose between using renewable raw materials or rerefined oil (min. 65 %). Additionally the metal cutting fluid must fulfil environmental requirements as to the base oil and the additives, while



environmental requirements are not imposed to transmission/gear fluids since requirements relating to toxicity, degradability and bioaccumulation are not compatible with the technical performance criteria.

The environmental criteria encompass that the components of the lubricant should have a high level and rate of biological degradability, have a low toxicity to waterborne organisms and should not have bioaccumulative potential ($\log P_{ow} < 3$ or $BCF < 100$). Specific criteria are determined for the base oil and for the additives:

- Base oil
 - Not classified with a N phrase (R50, R50/53, R51/53, R52/53 or R53)
 - Not classified as carcinogenic
 - Ready biodegradable (OECD 301 B, F, ISO 14593, ISO 9439 or ISO 9408)
 - Aquatic toxicity towards algae (OECD 201) and daphnia (OECD 202)
- Additives
 - If risk phrase R50 or R50/53
 - < 2 %: hydraulic oil & metal cutting fluid
 - < 1 %: chain oil, mould oil, 2-stroke oil & lubricating grease
 - If risk phrase R51/53
 - < 2 %: metal cutting fluid
 - < 1 %: chain oil, mould oil, hydraulic oil, 2-stroke oil & lubricating grease
 - If risk phrase R52/53 or R53
 - < 3 %: chain oil, mould oil & hydraulic oil
 - < 17%: lubricating grease
 - < 5 %: metal cutting fluid
 - < 15 %: 2-stroke oil
 - Aquatic toxicity towards algae (OECD 201) and daphnia (OECD 202)
 - No presence of endocrine disrupters
 - Aerosol products may not contain halogen hydrocarbon

In contrast to the other labelling systems, no fish toxicity is required. It is mentioned in the criteria document that fish toxicity is not required as fish have proved to be less sensitive than algae and Daphnia.



3.4.4 EU Ecolabel

The European Union has developed a label for products and services that have a reduced environmental impact throughout their complete life cycle, from the extraction of the raw material through production, use and disposal. The EU Ecolabel specifies criteria that reduce their environmental impact per product category. These criteria have been developed and agreed upon scientists, NGOs and stakeholders. The Ecolabel is considered to be the first major advancement towards creating a single international standard and it is becoming the most generally accepted label.

In order to obtain the EU Ecolabel, every product or service is carefully checked by independent experts. The scheme is voluntary, but currently already hundreds of companies across Europe have joined up. The logo of the EU Ecolabel is represented by a flower (Figure 14).



Figure 14. Logo of the EU Ecolabel.

The criteria in order to obtain the Ecolabel are specified per product category or per service category. There exists a specific category for lubricants. No specific category for solvents is described, but there exist product categories (for example “Indoor Paints & Varnishes”, “Outdoor Paints & Varnishes”, “Detergents” etc.), which will certainly contain solvents. Within the framework of this literature review also the criteria for outdoor paints and varnishes are described in order to obtain information with regard to solvents.



3.4.4.1 EU Ecolabel for lubricants

The aim of the EU Ecolabel for lubricants is promoting products that have a reduced impact on water and soil during use and contain a large fraction of bio-based material. The ecological criteria for the award of the EU Ecolabel to lubricants is described in the commission decision of 24 June 2011 (2011/381/EU).

The product group “lubricants” is subdivided into 5 categories:

- I. Hydraulic fluids and tractor transmission oils
- II. Greases and stern tube greases
- III. Chainsaw oils, concrete release agents, wire rope lubricants, stern tube oils and other total loss lubricants
- IV. Two-stroke oils
- V. Industrial and marine gear oils

Already a few products are certified according to the Ecolabel:

- Hydraulic fluids: 11 products
- Greases: 1 product
- Chainsaw oils: 5 products
- Two-stroke oils: 4 products
- Total loss lubricants: 4 products

The lubricants have to meet requirements for performance, show limited toxicity to aquatic organisms, have high biodegradability and a low potential for bioaccumulation and contain a high fraction of renewable raw materials. In order to obtain the EU Ecolabel for lubricants 7 criteria need to be fulfilled.

Criteria 1: Excluded or limited substances and mixtures (R-phrases)

The product or any part of the product may not contain substances meeting the criteria with the hazard statements or risk phrases (R-phrases) as described in the commission decision of 24 June 2011 (2011/381/EU). These include qualities such as carcinogenic potential, potential to cause birth defects, toxicity to aquatic life, hazards to ozone layer, etc. Moreover, it may not contain substances referred to in Article 57 of Regulation (EC) No 1907/2006. Concentration limits for substances meeting criteria of Article 57 (a), (b) or (c) of EC No 1907/2006 shall not exceed 0.010 % (w/w) and if specific concentration limits are referred to, they should remain below one tenth of the lowest specific concentration value unless this value falls below 0.010 % (w/w).

Criteria 2: Exclusion of specific substances

Substances, which are present in a concentration > 0.010 % (w/w) in the final product, may not be mentioned on the Union list of priority substances in the field of water policy. Moreover, no organic halogen compounds, nitrite compounds, metals and metallic compounds may be present in a concentration > 0.010 % (w/w). An exemption is made for sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) and for thickeners containing lithium (Li) or aluminium (Al) up to a certain concentration.



Criteria 3: Additional aquatic toxicity requirements

The aquatic toxicity needs to be evaluated for (1) the lubricant and its main components (= substances accounting for more than 5 % by weight of the lubricant) or for (2) each stated substance present above 0.10 % (w/w). Experience has learned that in nearly all current applications the second option is used.

In case that the lubricant and its main components are evaluated, acute aquatic toxicity data shall be provided for the lubricant on 3 trophic levels (algae, daphnia & fish), while for the main components data shall be provided on 2 trophic levels (algae & fish). Only (72hr) E_rC_{50} for algae (OECD 201, ISO 10253 or C.3 of EC No 440/2008¹³), (48hr) EC_{50} for daphnia (OECD 202, ISO TC 147/SC5/WG2 or C.2 of EC No 440/2008) and (96hr) LC_{50} for fish (OECD 203 or C.1 of EC No 440/2008) are accepted. The critical concentration for the acute aquatic toxicity for the lubricant is at least 100 mg/l for categories I or V or at least 1000 mg/l for categories II, III or IV. The critical concentration for the main components is at least 100 mg/l.

In case that each stated substance present above 0.10 % (w/w) is evaluated, chronic toxicity test results in the form of NOEC data shall be provided on 2 trophic levels (daphnia (C.20 of EC No 440/2008) and fish (C.14 of EC No 440/2008)). In case chronic toxicity results are missing, acute aquatic toxicity data shall be provided on 2 trophic levels (algae and daphnia). Only (72hr) E_rC_{50} for algae (OECD 201, ISO 10253 or C.3 of EC No 440/2008) and (48hr) EC_{50} for daphnia (OECD 202, ISO TC 147/SC5/WG2 or C.2 of EC No 440/2008) are accepted. The maximum cumulative mass concentration of substances exhibiting a certain degree of toxicity is given in Table 41.

If the substance is already stated on the Lubricant Substance Classification list or if a valid letter of a competent body can be submitted, no aquatic toxicity study needs to be conducted. Moreover, an aquatic toxicity is also not required if (1) the molecular weight is > 800 g/mol, (2) the molecular diameter is > 1.5 nm, (3) molecular weight fraction < 1000 g/mol of a polymer is less than 1 % or (4) substance is highly insoluble in water (< 10 µg/l) as such substances are not regarded as toxic for algae and daphnia in the aquatic system.

¹³ Council regulation EC No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation No 1907/2006 of the European Parliament and the Council of Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).



Table 41. Cumulative mass percentages (%w/w) of substances in the different lubricant categories with regard to aquatic toxicity.

Aquatic toxicity		Category				
		1	2	3	4	5
Not toxic	Acute toxicity > 100 mg/l OR NOEC > 10 mg/l			Not limited		
Harmful	10 mg/l < Acute toxicity ≤ 100 mg/l OR 1 mg/l < NOEC ≤ 10 mg/l	≤ 20	≤ 25	≤ 5	≤ 25	≤ 20
Toxic	1 mg/l < Acute toxicity ≤ 10 mg/l OR 0.1 mg/l < NOEC ≤ 1 mg/l	≤ 5	≤ 1	≤ 0.5	≤ 1	≤ 5
Very toxic	Acute toxicity ≤ 1 mg/l OR NOEC ≤ 0.1 mg/l	≤ 0.1 (M*)	≤ 0.1 (M*)	≤ 0.1 (M*)	≤ 0.1 (M*)	≤ 1 (M*)

* M is the multiplication factor of 10 for very toxic substances.

Criteria 4: Biodegradability and bioaccumulative potential

Only substances present in a concentration above 0.10 % (w/w) need to fulfill the requirements with regard to biodegradability and bioaccumulative potential. The minimum cumulative mass percentage of ultimately aerobically biodegradable substances and the maximum cumulative mass percentage of inherently aerobically biodegradable substances, non-biodegradable and non-bioaccumulative substances and non-biodegradable and bioaccumulative substances is given in Table 42.

Table 42. Cumulative mass percentages (%w/w) of substances in the different lubricant categories with regard to biodegradability and bioaccumulation.

Biodegradation and bioaccumulation		Category				
		1	2	3	4	5
Ultimately aerobically biodegradable		> 90	> 75	> 90	> 75	> 90
Inherently aerobically biodegradable		≤ 5		≤ 5	≤ 20	≤ 5
Non-biodegradable and non-bioaccumulative		≤ 5	≤ 25	≤ 5	≤ 10	≤ 5
Non-biodegradable and bioaccumulative		≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1

Test substances are considered ultimately aerobic biodegradable if at least 70 % biodegradation is reached based on dissolved organic carbon measurements within 28 days or if at least 60 % biodegradation is reached based on oxygen depletion or carbon dioxide



generation within 28 days (C.4 of EC No 440/2008 (OECD 301 A-F, OECD 306 or OECD 310) or if the BOD₅/ThOD or BOD₅/COD ratio is ≥ 0.5 (C.5 and C.6 of EC No 440/2008). For the evaluation of the biodegradation the principle of the 10-day window is not required.

Test substances are considered inherently biodegradable if a least 70 % biodegradation is reached in a test for inherently biodegradability (C.9 of EC No 440/2008 or OECD 302 C) or if a biodegradation percentage between 20 % and 60 % is obtained based on oxygen depletion or carbon dioxide generation within 28 days (C.4 of EC No 440/2008, OECD 306 or OECD 310).

A biodegradation test is not required if the substance, base fluid or additive is already stated on the Lubricant Substance Classification List or if a substance is not-biodegradable. Moreover, also read-across data may be used to estimate the biodegradability of a substance.

Bioaccumulation data are only required for those substances that are not ultimately nor inherently biodegradable. Test substances are considered not potential bioaccumulative if (1) the molecular weight is > 800 g/mol, (2) the molecular diameter is > 1.5 nm, (3) log K_{ow} value of < 3 or > 7 , (4) BCF ≤ 100 l/kg or (5) molecular weight fraction < 1000 g/mol of a polymer is less than 1 %.

Criteria 5: Renewable raw materials

The minimum carbon content derived from renewable raw materials is determined per product category. The minimum carbon content varies between 45 % (Category 2) and 70 % (Category 3) (Table 43).

Table 43. Minimum renewable content in the different lubricant categories.

Renewability	Category				
	1	2	3	4	5
Based on carbon	≥ 50 %	≥ 45 %	≥ 70 %	≥ 50 %	≥ 50 %

Criteria 6: Minimum technical performance

The minimum technical performances are determined per lubricant type.

Criteria 7: Information appearing on the EU Ecolabel

On the product an optional label may be used, which mentions that the product has a reduced harm for water and soil during use and that the product contains a large fraction of biobased material.

3.4.4.2 EU Ecolabel for outdoor paints and varnishes

The aim of the EU Ecolabel for outdoor paints and varnishes is to promote outdoor paints and varnishes, which are characterised by efficient use, a minimum of waste, a reduced environmental risk and other risks (by reducing the solvent emissions) and a reduced discharge of toxic or polluting substances into water. The ecological criteria for the award of



the EU Ecolabel to outdoor paints and varnishes is described in the commission decision of 13 August 2008 (2009/543/EC).

These criteria are related towards the white pigment content, the emissions and discharges of wastes from the production of any titanium dioxide pigment, a maximum volatile organic compounds (VOC) content, a maximum volatile aromatic hydrocarbon (VAH) content, heavy metals, restrictions towards the presence of dangerous substances and performance of the outdoor paints and varnishes.

The criteria towards dangerous substances are divided into different categories. The product may not be classified as very toxic, toxic, dangerous to the environment, carcinogenic, toxic for reproduction, harmful, corrosive, mutagenic or irritant. Moreover no ingredient classified as very toxic, toxic, carcinogenic, mutagenic and toxic for reproduction may be used according to a list of risk phrases or based on a list of categories of the Globally Harmonised System (GHS) of classification.

Moreover the use of alkylphenolethoxylates (APEOs), isothiazolinone compounds, perfluorinated alkyl sulfonates (PFAS), perfluorinated carboxylic acids (PFCA), formaldehyde, halogenated organic solvents and phthalates is forbidden or restricted.

Also the use of ingredients dangerous for the environment (defined with risk phrases ("dangerous for the environment") or on the GHS of classification) is restricted. GHS is the global (= worldwide) system for classification and labelling of chemical substances based on intrinsic hazardous properties. This system is developed by the United Nations. On European level, the CLP (= Classification, Labelling and Packaging of substances and mixtures) regulation (EC No 1272/2008 of 16 December 2008) modifies the existing European legislation in line with the regulation of GHS. This CLP regulations replaces the existing Dangerous substances directive (67/548/EG) and the Dangerous preparations directive (1999/45/EG). Especially with regard to the labelling significant modifications will be implemented: (1) signal words "Danger" and "Warning" will be implemented, (2) the R-phrases and the S-phrases will be replaced by H (= Hazard) statements and P (= Precaution) statements and (3) a new pictogram system will be applied. An overview of the existing risk phrases and categories based on the GHS of classification is given in Table 44.

Table 44. Overview of the existing classifications for environmental hazards.

Risk phrase	Description	GHS classification
N R50	Very toxic to aquatic organisms	Acute I
N R51	Toxic to aquatic organisms	Acute II
N R52	Harmful to aquatic organisms	Acute III
N R50-53	Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment	Chronic I
N R51-53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment	Chronic II
N R52-53	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment	Chronic III
N R53	May cause long-term adverse effects in the aquatic environment	Chronic IV



The basic elements used for classification of aquatic environmental hazards in both systems are based on acute aquatic toxicity, degradation, biodegradation and chronic aquatic toxicity. In Table 45 an overview is given of the criteria for the different GHS categories for substances.

Table 45. Overview of the criteria of the GHS classification system for environmental toxicity of substances.

GHS classification		
Acute I	96 hr LC ₅₀ (for fish) 48 hr EC ₅₀ (for crustacea) 72 or 96 hr ErC ₅₀ (for algae / other aquatic plants)	≤ 1 mg/l and/or ≤ 1 mg/l and/or ≤ 1 mg/l
Acute II	96 hr LC ₅₀ (for fish) 48 hr EC ₅₀ (for crustacea) 72 or 96 hr ErC ₅₀ (for algae / other aquatic plants)	>1 - ≤ 10 mg/l and/or >1 - ≤ 10 mg/l and/or >1 - ≤ 10 mg/l
Acute III	96 hr LC ₅₀ (for fish) 48 hr EC ₅₀ (for crustacea) 72 or 96 hr ErC ₅₀ (for algae / other aquatic plants)	>10 - ≤ 100 mg/l and/or >10 - ≤ 100 mg/l and/or >10 - ≤ 100 mg/l
Chronic I	96 hr LC ₅₀ (for fish) 48 hr EC ₅₀ (for crustacea) 72 or 96 hr ErC ₅₀ (for algae / other aquatic plants) AND Not rapidly degradable and/or Log K _{ow}	≤ 1 mg/l and/or ≤ 1 mg/l and/or ≤ 1 mg/l ≥ 4 (unless the BCF < 500)
Chronic II	96 hr LC ₅₀ (for fish) 48 hr EC ₅₀ (for crustacea) 72 or 96 hr ErC ₅₀ (for algae / other aquatic plants) AND Not rapidly degradable and/or Log K _{ow} Unless NOEC	>1 - ≤ 10 mg/l and/or >1 - ≤ 10 mg/l and/or >1 - ≤ 10 mg/l ≥ 4 (unless the BCF < 500) > 1 mg/l
Chronic III	96 hr LC ₅₀ (for fish) 48 hr EC ₅₀ (for crustacea) 72 or 96 hr ErC ₅₀ (for algae / other aquatic plants) AND Not rapidly degradable and/or Log K _{ow} Unless NOEC	>10 - ≤ 100 mg/l and/or >10 - ≤ 100 mg/l and/or >10 - ≤ 100 mg/l ≥ 4 (unless the BCF < 500) > 1 mg/l
Chronic IV	Poorly water soluble substances: No acute toxicity at levels up to water solubility AND Not rapidly degradable AND Log K _{ow} UNLESS BCF or NOEC	 > 4 < 500 > 1 mg/l



The approach for mixtures differs from the approach for substances. There exist different possibilities in order to classify mixtures.

- (1) When a mixture has been tested as a whole, it can be classified according to the criteria for substances for acute toxicity. It is not possible to classify a mixture for chronic classification because data from biodegradability and bioaccumulation of mixtures are only meaningful for single substances. If acute toxicity data are available for the mixture as a whole, these data and classification of the components for chronic hazard shall be used.
- (2) If there are no data available for the complete mixture, bridging principles can be used.
- (3) Classification can also be executed when data are available for all components or only for some components of the mixture. In this case the classification is based on the summation of the classification of its components taken into account the multiplication factor.



3.4.5 OK biodegradable WATER (Vinçotte)

The Belgian certification institute Vinçotte has developed a conformity mark for products, which are biodegradable in water (Figure 15).



Figure 15. OK biodegradable WATER logo (Vinçotte).

The criteria are partly based on the European specification EN 14987 (2006) towards plastics, which are disposable in waste water treatment systems. In order to obtain the OK biodegradable WATER conformity mark, the product must meet several criteria, which are related to biodegradability, solubility or dispersibility, ecotoxicity and chemical characteristics.

A product can only be approved on the condition that it is biodegradable (90% absolute or relative biodegradation) in water at ambient temperatures (20°C – 25°C) within 56 days (ISO 14851 or ISO 14852). Moreover, the product needs to be cold water soluble or cold water dispersible (EN 14987) and the heavy metals content and fluorine content must fulfil the requirements as prescribed by EN 13432. Furthermore, the product may not contain constituents that appear on the (candidate) list of Substances of Very High Concern.



3.5 Discussion and critical review

Based on the literature review of the different *biodegradation* test methods in an aqueous aerobic freshwater environment it can be concluded that a sufficiently broad range of measurement techniques already exists. The biodegradation rate can be determined based on the measurement of dissolved organic carbon, dissolved oxygen, CO₂ production, oxygen consumption or inorganic carbon. Not each test method is suitable to test bio-lubricants or bio-solvents due to the fact that bio-lubricants are often poorly water soluble and that bio-solvents are often volatile. Therefore specific biodegradation test method need to be selected taken into account these characteristics.

ASTM is the only organisation, that has developed specific biodegradation testing methods in an aqueous aerobic environment towards bio-lubricants. These methods are based on CO₂ production (ASTM D 5864 & ASTM D 6139) and oxygen consumption (ASTM D 6731). However, also OECD methods, international methods and European methods based on CO₂ production (OECD 301 B, ISO 9439 & EN ISO 9439), oxygen consumption (OECD 301 C, OECD 301 F, ISO 9408 & EN ISO 9408), dissolved oxygen (OECD 301 D, ISO 10707, EN ISO 10707 & ISO 10708) and inorganic carbon (OECD 310, ISO 14593 & EN ISO 14593) are appropriate to measure the biodegradation of bio-lubricants. Methods based on dissolved organic carbon are not suitable.

No specific standards were developed towards bio-solvents, but it is possible to use the already developed methods based on oxygen consumption (OECD 301 C, OECD 301 F, ISO 9408 & EN ISO 9408), dissolved oxygen (OECD 301 D, ISO 10707, EN ISO 10707 & ISO 10708) and inorganic carbon (OECD 310, ISO 14593 & EN ISO 14593) on condition that these methods are - if necessary - slightly adapted (= lower headspace) in order to minimize volatilisation. Methods based on dissolved organic carbon and CO₂ production (especially if the produced CO₂ is purged out of the system) are not appropriate for bio-solvents.

When testing poorly water soluble test items the addition of the test item to the test system needs to be adapted when compared to the addition of water soluble test items. Therefore the addition method as prescribed in the previous mentioned OECD, ISO and EN methods need to be modified. Such modifications are already described in ISO 10634, which was especially developed towards poorly water soluble test items.

The suitability of the proposed reference materials in the above recommended guidelines towards the testing of bio-lubricants and bio-solvents can be questioned. Reference materials are necessary in order to verify the activity of the inoculum. OECD 301 and the international standards for organic compounds propose aniline, sodium acetate and sodium benzoate as suitable reference materials. The main disadvantage of these materials is that they are very easily to degrade and even degraded when no inoculum is deliberately added (Aniline: Figure 16 & Sodium benzoate: Figure 17). Since the media in biodegradation tests are not sterilized, the distilled water contains also micro-organisms and these are often sufficient to degrade the proposed reference materials.



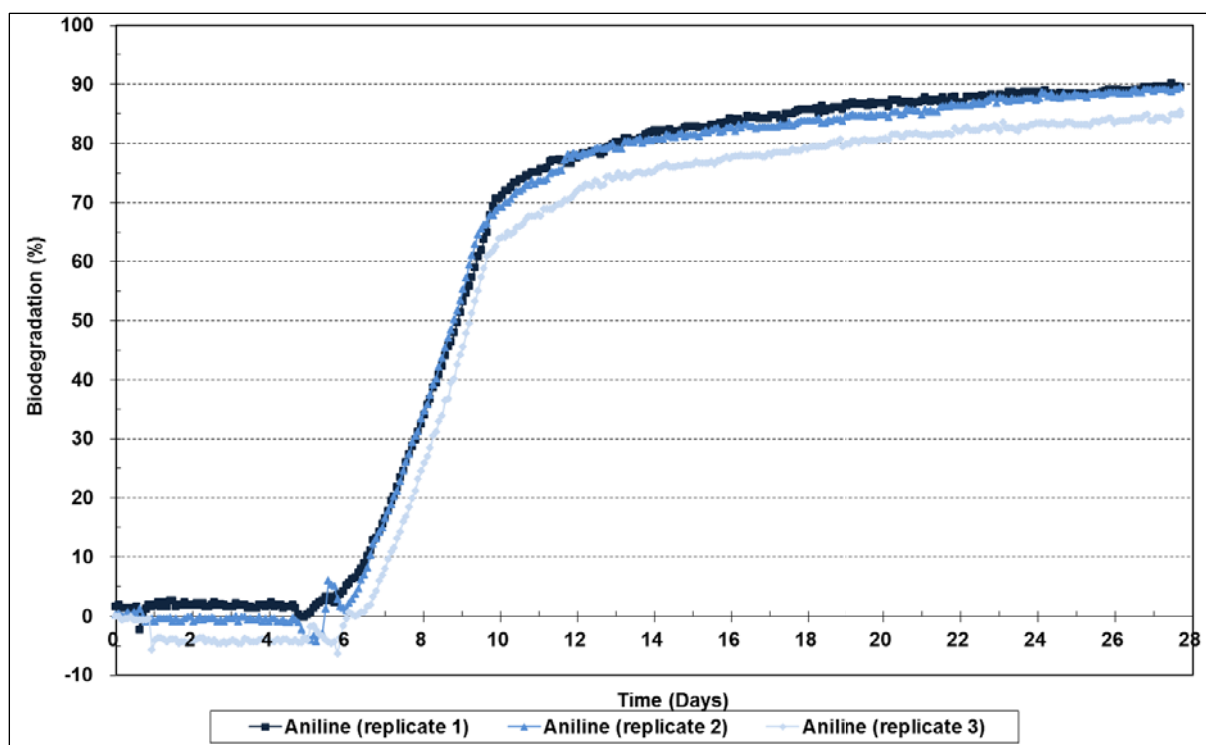


Figure 16. Evolution of the biodegradation percentage of 3 replicates of aniline (test system without inoculum based on the measurement of the oxygen consumption).

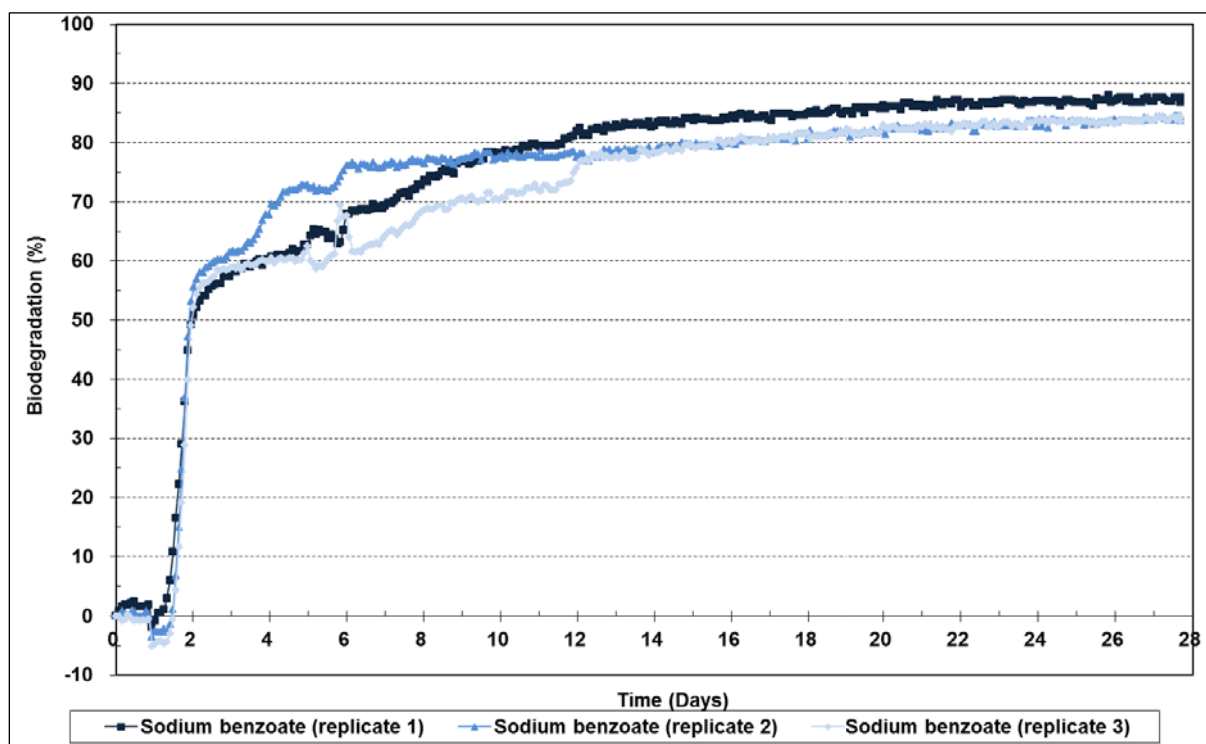


Figure 17. Evolution of the biodegradation percentage of 3 replicates of sodium benzoate (test system without inoculum based on the measurement of the oxygen consumption).



Consequently, objections can raise towards the use of these reference materials as they are not suitable in order to check the viability of the used inoculum. Therefore, it was suggested by OECD 301 to search another reference compound, which should be readily biodegradable but requires the addition of an inoculum. OECD investigated the suitability of potassium hydrogen phthalate (Figure 18) as ready biodegradable reference material, but at this moment no sufficient evidence is available.

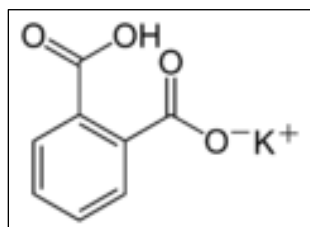


Figure 18. Structural formula of potassium hydrogen phthalate.

Another disadvantage of OECD 301 and the international standards towards organic compounds, is that they only refer to easy water-soluble chemicals as reference materials (Table 46).

Table 46. Solubility of reference materials in water (at a temperature of 20°C).

Reference material	Solubility
Aniline	34 g/l (Assink Chemie)
Sodium acetate	1190 g/l (Fisher Scientific)
Sodium benzoate	660 g/l (Chemical Book)

This can be considered as a deficit in the guidelines especially when testing bio-lubricants. OECD 310, which is a more recent guideline with regard to ready biodegradability of chemicals (2006), makes already a distinction between water-soluble (identical/comparable to OECD 301) and water-insoluble reference materials (1-octanol). However, it was demonstrated that 1-octanol also degrades without the presence of an inoculum (Figure 19). Also the American standards with regard to aerobic aqueous biodegradability of lubricants make a clear distinction between water-soluble (sodium benzoate and aniline) and water-insoluble (low erucic acid rapeseed oil) reference materials. The guidance document ECHA-12-G-22-EN for the implementation of REACH also mentions that the normal positive reference substances offer little support in the assessment of poorly soluble substances other than demonstrate that the inoculum is active (and even this can be questioned). It is suggested that in order to “bench mark” methods to assess poorly soluble substances common poorly soluble reference substances should be used (diisooctylphthalate or anthroquinone) (Figure 20) (ECHA (2012)).



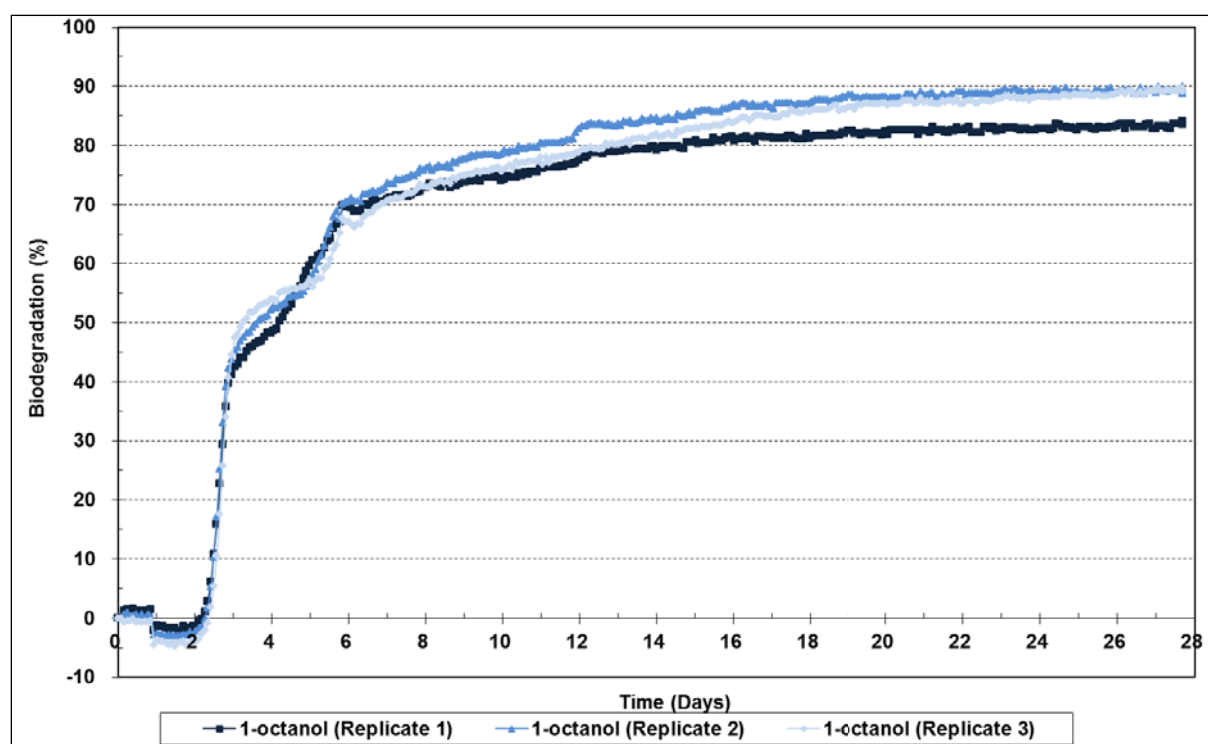


Figure 19. Evolution of the biodegradation percentage of 3 replicates of 1-octanol (test system without inoculum based on the measurement of the oxygen consumption).

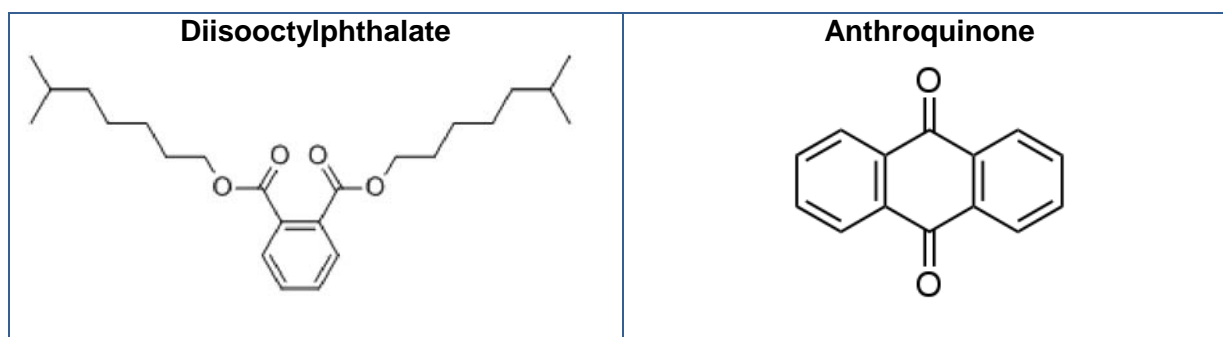


Figure 20. Structural formula of diisooctylphthalate or anthroquinone.

When testing bio-lubricants and bio-solvents, more appropriate reference materials should be chosen when compared to the currently proposed water soluble and very easily degradable reference materials as mentioned in the guidelines. A comparable system is already applied in biodegradation standards for plastics (ISO 14851 and ISO 14852). These standards still refer to a pure chemical (aniline) as reference material, but also more appropriate and challenging complex polymers (microcrystalline cellulose powder, ashless cellulose filters and poly- β -hydroxybutyrate) are mentioned as reference materials.

The biodegradation activity of the inoculum will be determined by various factors (geographical location of sampling, season of sampling, etc.). The major part of the standards refer to activated sludge, secondary effluent, surface water and soil as a suitable inoculum. It is expected that the diversity of the microbial flora and as such the



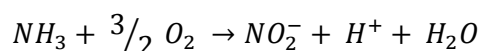
biodegradation potential will increase if more different sources are mixed in order to prepare the inoculum. Test methods OECD 301 C and OECD 302 C even prescribe that the micro-organisms need to be derived from at least 10 places (city sewage plants, industry sewage plants, rivers, lakes or sea). This will certainly increase the diversity of the bacterial community. However, after collecting the different sources, the inoculum is fed in the laboratory on a regular basis with synthetic sewage, which contains glucose, peptones and monopotassium phosphate. In spite of the fact that the microbial diversity at start is large, it is expected that the population of micro-organisms will lose their diversity due to the fact that they are always fed with the same food source.

The influence of the inoculum source on the biodegradation rate was evaluated in a study, which made a comparison between the biodegradation rate of cellulose, a biodegradable synthetic polyester (Mater-Bi NF01U) and poly(ϵ -caprolactone) in three different inoculum sources (sludge from a municipal wastewater-treatment plant in Novara, sludge from a municipal wastewater-treatment plant in Pero-Milan and sludge from an industrial membrane bioreactor treating wastewater from a chemical-pharmaceutical site). This study revealed that the origin of the inoculum is an important source of variability which affects the evaluation of the degree of mineralization. Sludges from different wastewater-treatment plants can be composed of different microbial populations and they can be characterized by different metabolic activities. Therefore, it was advised to define a “standard” inoculum, formed by mixing specific microbial strains conserved in microbial collections and known to possess, all together, all the relevant biochemical properties (Mezzanotte et al. (2005)).

Standardised inocula are already commercially available. A standardised inoculum could be a solution in order to increase reproducibility of test methods and this could reduce the dependence on seasonal and geographical variability. However, it still needs to be evaluated if they are divers enough in order to degrade difficult products. The diversity of standardised inocula should be compared to the diversity of natural inocula.

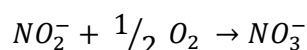
Another difficulty in the existing aerobic aqueous biodegradation test is related towards the high variation, which is sometimes observed for materials that require an adaptation phase. Test materials, which are either easily or poorly biodegradable, normally produce rather similar test results. On the other hand it is often noticed that the reproducibility of aqueous aerobic biodegradation tests on partly or moderately biodegradable substances is often very low, even if tests had been carried out in the same laboratory, by the same technicians, with the same test procedure and with the same batches of the same materials. Such substances often need special consortia of bacteria and long adaptation periods. More investigation is required in order to minimise the variation rate in aqueous aerobic freshwater biodegradation tests.

The variation in tests based on the oxygen consumption can be caused by nitrification. Nitrification takes place if ammonium salts and nitrogen-containing test compounds are oxidized to nitrite and nitrate (Equation 3 and Equation 4).



Equation 3. Nitrification reaction (conversion of ammonium to nitrite).





Equation 4. Nitrification reaction (conversion of nitrite to nitrate).

This reaction might cause interference in biodegradability assessments by reducing the pH value, which might inhibit bacterial action, and by taking up oxygen unpredictably, which can lead to problems in interpreting results of biodegradation tests based on oxygen consumption (OECD 301 C, OECD 301 F, ISO 9408, ISO 14851,...). If nitrification has occurred, the BOD values need to be corrected for interference by nitrification. In theory, it is expected that the nitrification process in the blank reactors, the reference reactors and the reactors with test item would be similar in the case the test item does not contain nitrogen. The international standards even mention that errors in case of nitrogen-free substances are normally negligible, because the oxidation of the ammonium in the medium is taken into account by the subtraction of the blank. In practice, however, it is often seen that this process shows large variations between the different reactors. This sporadic behaviour is typical for a reaction from micro-organisms which are relatively slow-growing and which are only present in a small minority in a population. Consequently, biodegradation curves based on oxygen consumption are often no correct reproduction of the biodegradation rate of a test item.

The nitrification process can be inhibited by the addition of specific inhibitors (e.g. allylthiourea, 2-chloro-6-trichloromethyl)pyridine, ...). ISO 9408 and ISO 14851 mention that allylthiourea can only inhibit nitrification during short incubation periods, as it is biodegradable. Therefore addition of allylthiourea to prevent nitrification is not recommended. It is mentioned that with low inoculum concentrations nitrification will not occur, even during long incubation periods without inhibitor. From the experience of OWS, it is known that nitrification often forms a problem for aerobic aqueous biodegradation tests based on oxygen consumption. Another option is to lower the ammonium concentration in the medium or to substitute it completely by nitrate. However, interferences cannot be completely eliminated (Painter, H.A. (1995)). Further research is required with regard to the suitability of tests based on oxygen consumption especially towards biodegradation tests with a long duration.

Taken into account that a large variation can be observed in aerobic freshwater tests, it would be better to test each series at least in triplicate (as suggested by the American aquatic biodegradability standards for lubricants) instead of the duplicates as recommended by OECD and ISO. Moreover, in order to ensure that the tests are reproducible, a validation criterion with regard to a maximum standard deviation between the replicates should be implemented in each test method (currently this is only the case for OECD guidelines).

ISO 15462 (2006) mentions that the criteria for readily biodegradability as described by OECD 301 seem to be too stringent for certain chemicals. Studies showed that products, which failed the criteria for ready biodegradability were, nevertheless, found to be adequately removed in simulation tests and in the real environment. Therefore, a recent European Regulation for testing of detergents no longer stipulates the 10-days window. The limit values (> 70 % for measurements based on DOC or > 60 % based on respirometric measurements)



should only be obtained within the 28 days period. This is also the case in the European Ecolabel for lubricants.

No specific criteria is given in ASTM D 5864, ASTM D 6139 and ASTM D 6731. These test methods state that if a test material achieves a high degree of biodegradation, it may be assumed that the test material easily biodegrades in many aerobic aquatic environments. However, no specific pass level ("high" degree: > 60 %, > 70 %, > 90 %?) nor a maximum duration are given. This should be clearly defined in order to avoid miscommunications.

Based on the literature review of the available standards with regard to **environmental safety**, it can be concluded that a sufficiently broad range of testing methods towards freshwater organisms on different trophic levels (bacteria, algae, freshwater aquatic plants, crustacean and fish) already exists. For bio-lubricants and bio-solvents, additional attention is especially needed towards the addition of poorly water soluble bio-lubricants and volatile bio-solvents to the testing systems as this can influence the test results. There exist already documents with specific guidance toward the sample preparation and the interpretation of the results for difficult substances (OECD), poorly water soluble substances (ISO 14442) and lubricants (ASTM D 6081). These guidelines should be taken into account when testing bio-lubricants and bio-solvents.

The literature review on the **specifications** revealed that currently no clear specification towards bio-lubricants and bio-solvents exists on European level. Therefore, there is an urgent need as standardisation can play an important role in the uptake of bio-based lubricants and bio-based solvents and consequently can help to increase market transparency by providing reference methods and criteria.

A technical report with useful recommendations for a specification towards bio-lubricants is already developed (CEN/TR 16227). This document and the specifications for the EU Ecolabel for lubricants can be taken as a guideline in order to develop a specification towards bio-lubricants. The document CEN/TR 16227 recommends that the minimum biodegradation percentage for oils is 60 % and the minimum biodegradation percentage for lubricating greases is 50 %. The biodegradation criterion as prescribed in the Ecolabel for lubricants is more progressive as it requires that the biodegradation of each component in a concentration > 0.1 % needs to be evaluated and the minimum biodegradation requirements (75 % or 90 %) refer to a minimum cumulative mass percentage that is ultimately biodegradable. This method prevents that a significant amount of non-biodegradable and potentially bioaccumulative substances are present in the lubricant. If only the final lubricant is evaluated for biodegradability and even if a biodegradation percentage of 60 % is obtained, it is still possible that a non-biodegradable and bioaccumulative component is present in the lubricant. This certainly needs to be taken into account when developing standard specifications for bio-lubricants. The system to evaluate biodegradation as mentioned by the Ecolabel can be considered as a good system to avoid that non-biodegradable and bioaccumulative substances are used in bio-lubricants. In the recommendation document and the specifications of the EU Ecolabel for lubricants no criteria are mentioned towards biodegradability and environmental safety in soil in spite of the fact that lubricants are often accidentally spilled in soil. Biodegradation and toxicity in a



soil environment are probably not required in order to avoid that it becomes too difficult for a product to fulfil the requirements. However, it might be possible that the biodegradation of some products proceeds slower in a soil environment when compared to a freshwater environment and it is possible that the lubricant concentration in soil is considerably higher than in water due less rapid dilution. Therefore, differences between biodegradation in soil and freshwater should be further investigated in order to be sure that this does not form a deficit in the standard specifications.

On international level a specification is developed towards environmentally acceptable hydraulic fluids (ISO 15380). The specification combines requirements with regard to readily biodegradability and aquatic environmental safety, but no criteria with regard to a minimum bio-based content nor biodegradation and/or toxicity in the soil environment are given. Moreover, the minimum biodegradation percentage (60 %) refers to the total lubricant and it is not mentioned that the biodegradability and the bioaccumulative potential of each component needs to be evaluated. As mentioned in the previous paragraph, this might allow the presence of non-biodegradable and bioaccumulative components in lubricants.

The review of the existing *labelling systems* revealed that there exist already different types of labelling systems towards bio-lubricants (EU Ecolabel for lubricants, German Der Blaue Engel ecolabel for chain lubricants for power saws (RAL-UZ 48), for lubricants and forming oils (RAL-UZ 64) and for hydraulic fluids (RAL-UZ 79)). The EU Ecolabel for lubricants is more progressive when compared to the German ecolabel as the EU Ecolabel has also specific criteria with regard to a minimum bio-based content. Although it must be noticed that the criteria with regard to renewability should not be too stringent as otherwise the industry will not be interested and the labelling system will not be used sufficiently (cfr. Nordic Swan ecolabel). It is remarkable that – in spite of the fact that bio-lubricants often contaminate soil – no biodegradation tests in soil nor toxicity tests on terrestrial organisms (earthworms or plants) are required.

No European or international labelling systems especially towards bio-solvents is developed yet. Following factors can be taken into account when developing a labelling system for bio-solvents:

- Biodegradability
- Source: bio-based
- Low environmental and human toxicity
- Performance
- No ozone depleting chemicals
- No global warming compounds
- No hazardous air pollutants
- Low or no volatile organic compounds
- Low vapour pressure
- High flash point

It is advised that a European or international labelling system towards bio-solvents should be developed in order to increase the market transparency.



4 Marine aerobic aqueous environment

The review of the marine aerobic aqueous environment has been executed by OWS.

4.1 Biodegradation

Initially biodegradation test methods in an aqueous medium were developed towards freshwater (Chapter 3.1). However, due to the growing awareness of the need to protect the marine environment against the increasing loads of chemicals, biodegradation methods were also developed for the marine environment. Biodegradation in a marine aerobic environment differs from biodegradation in a freshwater aerobic environment due to differences with regard to (1) the microbial population and (2) the chemical parameters of the water (salt content, nutrient content, etc.). Test conditions of marine environments are generally considered to be less favourable for biodegradation than those of limnic test systems.

Currently biodegradation test methods for a marine environment are developed on OECD level, ISO level and ASTM level. No European test method has been developed yet.

4.1.1 OECD guidelines

One OECD guideline with regard to the evaluation of the biodegradation of chemicals in seawater is developed yet: OECD 306 “Biodegradability in Seawater” (Adopted 17 July 1992). The results of this test give a first impression of biodegradability in seawater. If the results are positive ($> 70\%$ DOC removal or $> 60\%$ ThOD), it may be concluded that there is a potential for biodegradation in a marine environment. Although it must be noticed that this guideline is no simulation test as nutrients are added and the test concentration of the substance is much higher than the concentration that would be present in the sea. If a more definitive value would be required for the degree of biodegradation in seawater, other methods need to be used (e.g. simulation test in seawater using a test item concentration closer to the likely environmental concentration).

In this guideline two test methods are described: (1) the shake flask method and (2) closed bottle method. As the shake flask method is based on DOC measurements, this method is not very suitable in order to evaluate the biodegradation of bio-lubricants and bio-solvents. The closed bottle method, which is based on dissolved oxygen measurements, is more suitable for these substances.

An overview of the main parameters of these methods is given in Table 47, while the amount of replicates as prescribed by OECD 306 is given in Table 48.



Table 47. Overview of the main parameters as described in the shake flask method and the closed bottle method (OECD 306).

Parameter	Shake flask method	Closed bottle method
Suitable test items	Min. solubility: 25-40 mg C/l Not volatile No adsorption onto glass	Min. solubility: 2 mg/l (less soluble can also be tested)
Inoculum	Natural seawater (after filtration) to which nutrients are added (phosphate buffer, CaCl ₂ , MgSO ₄ ·7H ₂ O and FeCl ₃ ·6H ₂ O) DOC _{seawater} < 20% DOC _{test mixture}	Natural seawater (after filtration) to which nutrients are added (phosphate buffer, CaCl ₂ , MgSO ₄ ·7H ₂ O and FeCl ₃ ·6H ₂ O)
Temperature	15-20°C	
Reference material	Sodium benzoate, sodium acetate or aniline	
Measurement technique	DOC	Dissolved oxygen
Amount of test item	5-40 mg DOC/l	2-10 mg test substance/l
Duration	60 days Can be extended	28 days Can be extended on condition that the blank BOD values remain within the 30 % limit of the O ₂ in the test vessel (if this is not the case, results are not reliable due to interferences as wall growth and nitrification)
Validity	Reference substrate: comparable to results of ring test	Biodegradation reference substrate ≥ 60 % (short time span) & comparable to results of ring test Blank respiration < 30 % O ₂ test vessel

Table 48. Amount of replicates as prescribed by OECD 306.

Method		Blank series	Reference series	Test series	Abiotic sterile control	Toxicity control
Shake method	flask	2	1	2	1 (optionally)	1 (optionally)
Closed method	bottle	> 8	> 8	> 8	2 (optionally)	6



4.1.2 International standards

One ISO standard with regard to biodegradation of organic compounds in a marine environment is developed: ISO 16221 (2001) "Water quality – Guidance for determination of biodegradability in the marine environment". This standard is based on OECD 306, but a few modifications are made with regard to the measurement techniques and the inoculum. The measurement techniques are based on established aerobic freshwater tests. The main parameters of the test method are given in Table 49. The amount of replicates is in function of the used measurement technique (Table 50). For methods in which vessels have to be sacrificed for measurements more vessels are required.

Table 49. Overview of the main parameters as described in ISO 16221.

Parameter	Description
Inoculum	Natural seawater (after filtration) (bacterial concentration: $\pm 10^5$ cells/ml) with nutrients (phosphate buffer & $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) or artificial seawater
Temperature	15-25°C
Reference material	Sodium benzoate or aniline
Measurement technique	DOC die-away test (ISO 7827) (DOC measurements) Closed bottle test (ISO 10707) (BOD measurements) Two-phase closed bottle test (ISO 10708) (BOD measurements) CO ₂ evolution test (ISO 9439) (CO ₂ measurements) CO ₂ headspace test (ISO 14593) (TIC measurements)
Amount of test item	5-40 mg DOC/l (ISO 7827) 2-10 mg substance/l (ISO 10707) 100 mg ThOD/l (ISO 10708) 20 mg TOC/l (ISO 9439) 20-40 mg TOC/l (ISO 14593)
Duration	60 days
Validity	Biodegradation reference material > 60 % (respirometric measurements) or > 70 % (DOC measurements) after 14 days

Table 50. Minimum amount of replicates as prescribed by ISO 16221.

Method	Blank series	Reference series	Test series	Abiotic sterile control	Toxicity control
ISO 16221	2	1	2	1 (optionally)	1 (optionally)

At this moment ISO is developing a new method in order to determine the aerobic biodegradation of plastics sunk at the sea water / sandy sediment interface. This method will simulate a habitat found in the benthic zone where sunlight reaches the ocean floor (= photic zone = sublittoral zone). The proposed test set-up consists of a solid phase (= sandy sediment) and a liquid phase (= synthetic seawater) in a closed, unstirred respirometer incubated at 20°C – 28°C. The film sample, as a disk, is put on the sediment and covered by a coverslip. Biodegradation is measured by oxygen consumption. This test method is based on the article written by Tosin et al. (2012).



4.1.3 American standards

An overview of the American standards with regard to biodegradation and weight attrition in a marine environment is given in Table 51. ASTM D 6691 and ASTM D 7473 are both referring to plastics, but ASTM D 6691 can be described as a Tier 1 test, while ASTM D 7473 is a Tier 2 test, closer to real-life conditions. In ASTM D 6691 the sample is cryogenically milled to increase the surface area and biodegradability (CO_2 production) is determined, while plastics are tested as such in ASTM D 7473 and weight loss is measured. As weight loss (= disintegration = physically fallen apart into smaller pieces) is measured, this standard cannot be used for demonstrating biodegradation (= complete mineralisation to H_2O , CO_2 and biomass). ASTM D 6692 is designed in order to determine the degree of aerobic biodegradability of polymeric compounds utilized in plastic materials by determining the level of respiration of such radiolabeled carbon compounds to radiolabeled carbon dioxide.

No specific American standards with regard to the biodegradation of lubricants or solvents are available.

Table 51. Overview of the ASTM standards with regard to biodegradation in aerobic marine environment.

Standard	Description
D 6691 - 09	Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in the Marine Environment by a Defined Microbial Consortium or Natural Sea Water Inoculum
D 6692 - 01	Standard Test Method for Determining the Biodegradability of Radiolabeled Polymeric Plastic Materials in Seawater
D 7473 - 12	Standard Test Method for Weight Attrition of Plastic Materials in the Marine Environment by Open System Aquarium Incubations



The main principles of the American standard test methods D 6691 and D 6692, which determine the biodegradability of plastic materials in marine environments, are given in Table 52.

Table 52. Overview of the main parameters as described in ASTM D 6691 – 09 and ASTM D 6692- 01.

Parameter	ASTM D 6691 - 09	ASTM D 6692 - 01
Inoculum	Synthetic seawater with pre-grown population of at least 10 aerobic marine micro-organisms Natural seawater with inorganic nutrients (0.5 g/l NH ₄ Cl & 0.1 g/l of KH ₂ (PO ₄))	Natural sea water with inorganic nutrients (0.5 g/l NH ₄ Cl & 0.1 g/l of KH ₂ (PO ₄)) Marine sediment can be added to increase the microbial diversity
Temperature	30 ± 2°C	Constant temperature (no specific temperature is mentioned)
Reference material	Cellulose, chitin or Kraft paper (control for activity of the inoculum) Sodium bicarbonate and sodium sulfite (control for CO ₂ sensors)	Glucose or starch (uniformly labeled by ¹⁴ C)
Measurement technique	Respirometer to measure the CO ₂ production	Measurement of amount of radioactive polymer that had been mineralized to ¹⁴ CO ₂ at various time points. Bottles are sacrificed at measurement. Before measuring the produced CO ₂ , the pH of the samples is brought to 2.5-3 followed by 6 hours shaking. During this period the ¹⁴ CO ₂ is trapped in a filter paper wick with an appropriate CO ₂ trapping agent.
Sample bottle	125 ml bottles	120 ml serum bottles
Amount of test item	20 mg per bottle	5-10 mg uniform ¹⁴ C radiolabeled polymer per bottle (Specific activity > 0.1 µCi/mg and < 5-10 µCi/mg)
Duration	Normally 10 – 90 days	Several days to several weeks
Amount of replicates	Triplicate	At least 6 bottles per series
Validity	Reference > 70 % biodegradation	No validation criteria



In spite of the fact that ASTM D 7473 is no standard test method with regard to biodegradability, the main principles of this standard is discussed in this chapter due to the relationship with ASTM D 6691. ASTM D 7473 is used to measure the weight loss as a function of time for non-floating plastic materials under continuous flow (open system) aquarium conditions. The conditions as simulated in this test are representative for aquatic environments near the coasts and near the bottom of a water body in absence of sunlight. Aquarium testing is considered as a more realistic approach of a marine environment when compared to a closed flask test as an aquarium test allows flushing, exposure to a diverse population of microbes, removal of metabolic end products, re-supply of oxygen, exposure to anoxic conditions in sediment and exposure to seasonal temperature variation of incoming seawater and natural concentration of macro- and micronutrients.

This test method can only be applied on materials, which achieve at least 30 % mineralization in test method ASTM D 6691. If a test material does not reach 30 % mineralization according to ASTM D 6691, it shall be considered non-biodegradable in the marine environment. The main parameters of test method ASTM D 7473 are given in Table 53.

Table 53. Overview of the main parameters as described in ASTM D 7473 - 12.

Parameter	ASTM D 7473 - 12
Inoculum	(1) Continuous fresh supply of natural seawater (= oxygenated seawater) (2) Continuous fresh supply of natural seawater (= oxygenated seawater) & surface marine sediment (anaerobic processes can play a role for films placed on the sediment)
Temperature	Temperature of the natural seawater is recorded at zero time and at each sampling point. Seasonal temperature fluctuations and mesophilic and psychrophilic microbes will play a role.
Reference material	-
Measurement technique	At selected time intervals, samples (triplicate) are removed from the aquarium box. The samples are rinsed and the weight of the rinsed samples is determined after drying to constant weight (35-40°C). The samples are also inspected visually (for example: blackening of the undersides of the sample). Correction is made for soluble components. Weight loss is also calculated per unit area of film.
Sample bottle	Plastic boxes
Amount of test item	0.5 by 0.5 inch pieces
Duration	180 days
Amount of replicates	3 replicates per weight determination / 5 weight determinations per test
Validity	-



4.2 Toxicity

4.2.1 Preparation of the test items

The preparation of poorly soluble or volatile substances is in detail described in chapter 3.2.1.

4.2.2 Test methods

4.2.2.1 OECD guidelines

OECD has developed several guidelines for freshwater species (chapter 3.2.2.1). The major part of the fish toxicity tests were also developed towards freshwater fish species, but in OECD 210 also a marine fish species is recommended in order to execute the test (Sheepshead minnow (*Cyprinodon variegatus*)).

4.2.2.2 International standards

ISO has already developed growth inhibition tests towards marine algal species (*Skeletonema costatum*, *Phaeodactylum tricornutum* and *Ceramium tenuicorne*) (Table 54). The principle of these tests are comparable to the growth inhibition test on freshwater alga as prescribed in OECD 201, but these marine toxicity tests need to be executed in natural or synthetic seawater to which nutrients are added.

Table 54. Overview of the international standards with regard to marine aquatic toxicity.

ISO standard	Description
10253 (2006)	Water quality – Marine algal growth inhibition test with <i>Skeletonema costatum</i> and <i>Phaeodactylum tricornutum</i>
10710 (2010)	Water quality – Growth inhibition test with the marine and the brackish water macroalga <i>Ceramium tenuicorne</i>

4.2.2.3 American standards

ASTM has developed a broad range of toxicity tests on different marine species (luminescent marine bacterium, saltwater bivalve molluscs, saltwater mysids, estuarine and marine invertebrates, polychaetous annelids, echinoid, bioluminescent dinoflagellates, meiobenthic copepod). An overview of the available standards is given in Table 55.

Table 55. Overview of the ASTM standards with regard to marine aquatic toxicity.

Standard	Description
D 5660 – 96 (2009)	Standard Test Method for Assessing the Microbial Detoxification of Chemically Contaminated Water and Soil Using a Toxicity Test with Luminescent Marine Bacterium
E 724 – 98 (2004)	Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs
E 1191 – 03a (2008)	Standard Guide for Conducting Life Cycle Toxicity Tests with Saltwater Mysids
E 1367 – 03 (2008)	Standard Test Method for Measuring the Toxicity of Sediment Associated Contaminants with Estuarine and Marine Invertebrates



Standard	Description
E 1463 – 92 (2004)	Standard Guide for Conducting Static and Flow Through Acute Toxicity Tests With Mysids from the West Coast of the United States
E 1562 – 00 (2006)	Standard Guide for Conducting Acute, Chronic and Life Cycle Aquatic Toxicity Tests with Polychaetous Annelids
E 1563 – 98 (2004)	Standard Guide for Conducting Static Acute Toxicity Tests with Echinoid Embryos
E 1611 – 00 (2007)	Standard Guide for Conducting Sediment Toxicity Tests with Polychaetous Annelids
E 1924 – 97 (2004)	Standard Guide for Conducting Toxicity Tests with Bioluminescent Dinoflagellates
E 2317 – 04	Standard Guide for Conducting Renewal Microplate Based Life Cycle Toxicity Tests with a Marine Meiobenthic Copepod



4.3 Standard specifications

Standard specifications especially towards bio-lubricants or bio-solvents are not yet developed for a marine environment.

The *Convention for the Protection of the Marine Environment of the North-East Atlantic* (OSPAR Convention) is the current legal instrument guiding international cooperation on the protection of the marine environment of the North-East Atlantic. The OSPAR Commission is made up by representatives of 15 contracting Governments and the European Commission.

Offshore chemicals that are identified by one of following criteria shall be substituted if a less hazardous (or preferably non-hazardous) substitute is available:

- Listed in the OSPAR List of Chemicals for Priority Action
- Substances considered by the authority to be of equivalent concern for the marine environment as the above mentioned category
- Inorganic combined with high toxicity
- Persistent
- Meet two of following criteria:
 - Not readily biodegradable
 - High bioaccumulation potential
 - High toxicity

The OSPAR guidelines for environmental compliance require component level testing of chemicals released to the marine environment for biodegradation, bioaccumulation and toxicity. These standards are considered to be the most appropriate for measuring the overall impact of a substance in the marine environment (United States Environmental Protection Agency, 2011). Following criteria need to be evaluated for each component:

- Persistence (biodegradation in seawater according to OECD 306)
- Bioaccumulation (K_{ow})
- Marine toxicity
 - Growth inhibition test using the marine alga *Skeletonema costatum*
 - Acute toxicity test using the marine copepod *Acartia tonsa*
 - A sediment bioassay using an amphipod *Corophium sp*
 - A fish acute toxicity test (recommendation: turbot juvenile)



ASTM D 7081 is a standard specification, which encompasses criteria (including disintegration, biodegradation and environmental impacts with regard to aquatic toxicity, metals and other toxic substances) for non-floating plastics that are designed to be biodegradable under the marine environmental conditions of aerobic marine waters or anaerobic marine sediments. This standard specification is intended to establish the requirements for labelling materials and products, as “biodegradable in marine waters and sediments” or “marine disposable”. An overview of the specific requirements is given in Table 56.

Table 56. Overview of the requirements for biodegradable plastics in marine waters and sediments as prescribed by ASTM D 7081 - 05.

Parameter	Requirement as prescribed by ASTM D 7081 - 05
Density	Minimum 1.05 g/cm ³
Disintegration	Monomaterial: Maximum 30 % remains in the > 2 mm fraction after 12 weeks (ASTM D 6691 under mesophilic or psychrophilic conditions)
Biodegradation	<p>Minimum 30 % relative biodegradation (ASTM D 6691) within 180 days at 30 ± 2°C</p> <p>Minimum 90 % biodegradation in an active environment (compost) (ASTM D 5338)</p> <p>Satisfactory rate of biodegradation in test method ASTM D 5338 within 180 days or 365 days (for radiolabeled materials):</p> <ol style="list-style-type: none"> 1. Homopolymer: minimum 60 % relative biodegradation OR 2. Other polymers and substrates: minimum 90 % relative biodegradation OR 3. Products (> 1 polymer): each polymer in a concentration > 1 %: 60 % relative biodegradation <p>Remark: Plastics used as coating or binder, need to be tested separately and need to reach 90 % biodegradation</p>
Toxicity	<p>Products shall not adversely impact on the survival of marine organisms nor adversely affect the ecosystem using one of following methods:</p> <ol style="list-style-type: none"> 1. Polytox (microbial oxygen absorption) 2. Microtox (microbial bioluminescence) 3. Fish acute toxicity (OPPTS 850.1075) 4. Daphnia acute toxicity (OPPTS 850.1010) 5. Static algal toxicity (OPPTS 850.5400) <p>Heavy metals < 25 % of those prescribed in the country where the product is sold</p>
Others	Compliance to ASTM D 6400



4.4 Labelling

The product group “lubricants” as described in the chapter with regard to labelling systems related to freshwater aerobic aqueous environments (Chapter 3.4) often contains applications for the shipping industry. For example:

- EU Ecolabel for lubricants
 - Category 2: Stern tube greases
 - Category 3: Stern tube oils
 - Category 5: Marine gear oils

In the EU Ecolabel for lubricants it is not specified that a marine biodegradation test or environmental safety towards marine species must be evaluated if the product is used in a marine environment. However, the commission decree specifies that besides a freshwater biodegradation test (Part C.4 of the Annex to Regulation (EC) No 440/2008 (= DOC Die-Away, Modified OECD Screening DOC Die-Away, CO₂ Evolution, Manometric Respirometry, Closed Bottle or MITI) or OECD 310) a marine biodegradation test (OECD 306) can be used in order to determine if a substance is ultimate biodegradable or inherently biodegradable.

In the European Ecolabel application pack for lubricants it is mentioned that the applicant will select the most appropriate test method according to the intended use for the candidate lubricant, marine or freshwater applications (e.g. in a marine environment the biodegradation is established in marine water). It is also mentioned in the European Ecolabel application pack for lubricants that both sea and freshwater toxicity tests are allowed.

Consequently, it is possible to evaluate biodegradability and aquatic toxicity in a marine environment for marine applications.



4.5 Discussion and critical review

From the literature review on the *biodegradation* test methods in a marine aerobic environment, it can be concluded that there exist considerably less biodegradation test methods when compared to a freshwater aerobic environment. However, it can still be concluded that a sufficiently broad range of methods exists in order to determine the biodegradation in a marine environment.

Not all methods are suitable in order to evaluate the biodegradability of bio-lubricants and bio-solvents, but a suitable measurement technique can be selected taken into account the properties (volatility and/or solubility) of the bio-lubricant or the bio-solvent. Following methods are normally appropriate:

- OECD 306 (closed bottle test)
- ISO 16221 (measure method based on ISO 10707, ISO 10708, ISO 9439 (only for bio-lubricants) or ISO 14593)

Comparable to the freshwater biodegradation tests, the addition method of the poorly soluble and volatile substances to the test reactors needs to be adapted when compared to the addition of water soluble test items. Such modifications are already described in ISO 10634, which was especially developed towards poorly water soluble test items.

In spite of the fact that all methods measure biodegradation in a marine environment, there exist differences with regard to the preparation of the seawater inoculum. OECD 306 only refers to natural seawater, while ISO 16221 allows the use of natural seawater and artificial seawater. If natural seawater is used as inoculum, seasonal and geographic variations will influence the biodegradation activity. Moreover, it is noticed that the addition of inorganic nutrients to the natural seawater also varies between the different methods. OECD 306 prescribes that a phosphate buffer, CaCl_2 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ is added to the seawater, while only the inorganic nutrients phosphate buffer and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ are allowed by ISO 16221. The nutrient availability can also influence the biodegradation results. Further research should be executed in order to reduce inoculum variability.

The developed biodegradability test methods simulate aerobic environments in seawater. Tosin M. et al. (2012) suggests that a test methodology with regard to biodegradability of bioplastics in a marine environment should be developed per different habitat (supralittoral, eulittoral, sublittoral benthic, deep sea benthic, pelagic & buried in the sediments) as there exist large differences with regard to light, oxygen concentration, microbial community, friction and nutrient concentrations between these environments. Further research should be executed in this area.

From the review of the standards with regard to *marine environmental safety* it can be concluded that less tests were developed when compared to freshwater environment safety especially on OECD level. ISO and ASTM are already more progressive as more guidelines towards marine organisms were developed. The guidance documents towards the sample preparation and the interpretation of the results of toxicity tests for difficult substances (OECD), poorly water soluble substances (ISO 14442) and lubricants (ASTM D 6081) should be taken into account.



Currently no *standard specifications* towards more environmentally friendly alternatives for lubricants and solvents used in a marine environment are developed yet.

The *EU Ecolabel system* for lubricants described in the chapter with regard to the freshwater environment (Chapter 3.4.4.1) also covers applications in the shipping industry in a marine environment (stern tube lubricants, etc.). The European Ecolabel application pack for lubricants mentions that the applicant needs to select the most appropriate test method according to the intended use for the candidate lubricant (e.g. in a marine environment the biodegradation is established in marine water). Due to differences between a freshwater and a marine environment, it is indeed better to execute the tests in a marine environment. This should also be recommended in the commission decision of 24 June 2011 (2011/381/EU). If it is allowed that both freshwater and marine tests can be used in order to obtain an Ecolabel for marine applications, more research should be executed, which compares results of freshwater tests with results of marine tests.

The application of the marine test environment for organic substances, which might be accidentally spilled in the sea, is already clearly defined in the regulation of the OSPAR Convention, which requires that both biodegradation and environmental safety are tested using marine biodegradation and marine toxicity methods, and in the American standard specification for non-floating biodegradable plastics in a marine environment (ASTM D 7081), which requires that biodegradation needs to be tested in a marine environment (ASTM D 6691). ASTM D 7081 requires that a high biodegradation percentage (60 % for homopolymers or 90 % for other polymers) needs to be obtained in a biodegradation test in a compost environment, but the biodegradation criterion in a marine environment (only 30 %) as prescribed by this standard specification is very low. For example: a plastic material composed of 50 % component A, which is only biodegradable at high temperatures, and 50 % component B, which is biodegradable at high and low temperatures, will easily reach the 90 % biodegradation criterion in a controlled composting environment at 58°C and will also easily reach 30 % biodegradation criterion in a marine environment due to the degradation of component B. However, as component A needs a thermal trigger in order to biodegrade, component A will not be degraded in a marine environment. Such materials are already on the market. This criterion should better be revised. For the evaluation of the toxicity, it is not required that the proposed test are executed using marine species.

